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Constraint-based modelling of mixed microbial populations: Application to polyhydroxyalkanoates production

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"Biologists can be divided into two classes: experimentalists who observe things that cannot be explained, and theoreticians who explain things that cannot be observed"

Aharon Katzir-Katchalsky, 1982

Abstract

The combined use of mixed microbial cultures (MMC) and fermented feedstock as substrate may significantly decrease polyhydroxyalkanoates (PHA) production costs and make them more competitive in relation to conventional petroleum-based polymers. However, there still exists a lack of knowledge at metabolic level that limits the development of strategies to make this process more effective. In this thesis, system biology computational tools were developed and applied to PHA production by MMC from fermented sugar cane molasses, rich in volatile fatty acids (VFA). Firstly, a metabolic network able to describe the uptake of complex mixtures of VFA and PHA production was defined. This metabolic network was applied to metabolic flux analysis (MFA) to describe substrate uptake and PHA production fluxes over the enrichment time of a culture submitted to the feast and famine regimen. Then, the minimization of the tricarboxylic acid cycle (TCA) fluxes was identified as the key metabolic objective of a MMC subjected to this regimen by flux balance analysis (FBA). This model enabled to predict, with an acceptable accuracy, the PHA fluxes and biopolymer composition. Subsequently, data gathered from microautoradiography-fluorescence in situ hybridization (MAR-FISH) was used to develop a segregated FBA model able to predict the flux distribution for the three populations identified in the enriched culture. These results were slightly better than those obtained by the non-segregated FBA and were consistent with MFA results. Finally, a dynamic metabolic model was proposed based on the previous models and on a regulatory factor for VFA uptake and PHA production. This model allowed to identify the dynamics of the process and regulatory factor as well as to validate the previous results. Globally, this thesis enabled to demonstrate the potential of using computational tools to understand and optimize PHA production by MMC.

Keywords: *polyhydroxyalkanoates (PHA); mixed microbial culture (MMC); fermented feedstock; constraint based modelling; flux balance analysis (FBA); metabolic flux analysis (MFA)*

Resumo

A utilização combinada de culturas microbianas mistas (MMC) e substratos de baixo valor comercial poderá reduzir significativamente os custos de produção de polihidroxialcanoatos (PHA) e torná-los competitivos relativamente aos polímeros sintéticos convencionais. Contudo, existe ainda falta de conhecimento a nível metabólico que limita o desenvolvimento de estratégias para um processo mais eficiente. Na presente tese, desenvolveu-se e aplicou-se ferramentas computacionais de biologia de sistemas a processos de produção de PHA por MMC utilizando melaços de cana de açúcar fermentados ricos em ácidos orgânicos voláteis (VFA). Começou-se por definir uma rede metabólica para descrever o consumo de misturas complexas de VFA para a produção de PHA. Aplicou-se esta rede numa análise de fluxos metabólicos (MFA), que permitiu descrever os fluxos de consumo de substratos e de acumulação de PHA ao longo do período de enriquecimento da MMC sujeita a um regime de fartura/fome. De seguida, utilizando o balanço de fluxos (FBA), determinou-se que a minimização dos fluxos relativos ao ciclo do ácido cítrico (TCA) é o objectivo metabólico chave de uma MMC. Com este modelo identificou-se com precisão os fluxos de produção e a composição dos PHA. Posteriormente, utilizou-se os dados provenientes de MAR-FISH (Microautoradiography-Fluorescence in Situ Hybridization) para desenvolver um modelo segregado capaz de prever a distribuição de fluxos para as três populações identificadas na cultura enriquecida. Estas previsões foram ligeiramente melhores do que as obtidas pelo FBA não segregados e concordantes com os resultados obtidos por MFA. Para finalizar, desenvolveu-se um modelo metabólico dinâmico baseado nos modelos anteriores e num factor de regulação para o consumo de VFA e produção de PHA. Este modelo permitiu identificar a dinâmica do processo e do factor de regulação e validar os resultados anteriores. Globalmente, demonstrou-se o potencial de utilização de ferramentas computacionais na compreensão/optimização do processo de produção de PHA por MMC.

Palavras chave: *polihidroxialcanoatos (PHA); culturas microbianas mistas (MMC, 'mixed microbial cultures'); substratos de baixo valor comercial; modelação baseada em restrições; análise de balanço de fluxos (FBA, 'flux balance analysis'); análise de fluxos metabólicos (MFA, 'metabolic flux analysis')*

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List of Notations and Abbreviations

Φ:	Regulation factor
Φ':	Regulation factor affecting substrates ($\Phi' = \Phi$)
Φ'':	Regulation factor affecting products ($\Phi'' = 1/\Phi$)
A:	matrix of metabolic fluxes
A_b:	matrix of known fluxes
Ac:	Acetate
$A_n^{\#}$:	pseudo inverse matrix of unknown fluxes
A_n:	matrix of unknown fluxes
ATP:	Adenosine-5'-TriPhosphate
But:	Butyrate
CO_2:	Carbon dioxide
CSTR:	Continuous Stirred Tank Reactor
DOC:	Dissolved Organic Carbon
E_FA:	Unknown Fatty acids with even number of carbon atoms
Even:	All carbon sources with even number of carbon atoms
FA:	Fatty Acids (sum of E_FA with O_FA)
f_i:	intracellular PHA, PHB and PHV contents (C-mol/C-mol)
FISH:	Fluorescence in situ hybridization
FM:	fermented sugar cane molasses
$f_{PHA,max}$:	maximum intracellular PHA contents (C-mol/C-mol)
f_{ri}:	normalized intracellular carbon flux of reaction i (C-mol i/C-mol DOC) (the normalization was done by total carbon uptake (r_s))
f_X:	fraction of each population (Azoarcus, Thauera and Paracoccus)
GPC-SEC:	Gel Permeation Chromatography - Size Exclusion Chromatography
h:	consistency index
HA:	Hydroxyalkanoate
HB:	Hydroxybutyrate
HV:	Hydroxyvalerate
j:	index of each population (1 – Azoarcus, 2 – Thauera and 3 – Paracoccus)
K_N:	ammonia half-saturation constant (N-mmol/L);
K_S:	substrate half-saturation constants (C-mmol/L)
MAR-FISH:	Microautoradiography-Fluorescence in situ hybridization

m_{ATP} :	maintenance on ATP [mol ATP/(C-mol.h)]
mcl:	medium-chain length
MFA:	Metabolic Flux Analysis
MFD:	Metabolic Flux Distribution
MMC:	Mixed Microbial Cultures
MW:	molecular weight
NAD(P)H/NAD(P):	Reduced form/protonated form of Nicotinamide adenine dinucleotide (phosphate)
$NADH_2$:	Reduced form of Nicotinamide adenine dinucleotide (phosphate)
n_i:	number of carbon atoms for *i* substrate
O_FA:	Unknown Fatty acids with odd number of carbon atoms
O_2:	Oxygen
Odd:	All carbon sources with odd number of carbon atoms
P/O ratio, δ:	oxidative phosphorylation efficiency (mol-ATP/mol-$NADH_2$)
PHA:	Polyhydroxyalkanoates
PHB:	Polyhydroxybutyrate
PHV:	Polyhydroxyvalerate
Prop:	Propionate
r:	vector of metabolic fluxes
$r_{i,max}$:	maximum specific rate of reaction on compound i [C-mol/(C-mol.h)]
R_i:	reaction name
r_i:	specific rate of reaction on compound i [C-mol/(C-mol.h)]
SBR:	Sequencing Batch Reactor
SRT:	Sludge Retention Time
TCA:	Tricarboxylic Acid
TOC:	Total organic carbon
v:	vector of metabolic fluxes
Val:	Valerate
v_b:	vector of known fluxes
VFA:	Volatile Fatty Acids
v_i:	flux of Ri reaction [C-mmol/(C-mmol X.h)]
v_n:	vector of unknown fluxes
X:	active biomass
α:	saturation order constant
χ^2:	Chi-square distribution

1 *Thesis motivation and outline*

1.1. Thesis Motivation

Nowadays, all of us are surrounded by numerous polymeric materials (fabrics, electronic devices, construction materials, commodity daily use containers like bottles and bags, etc). Over the last decades these materials contributed greatly to the improvement on quality of life and to the use of resources more efficiently. However, the vast majority of these polymeric materials are synthetic polymers derived from fossil fuels resources. This is a major economical problem due to the decrease of availability of the natural resources required for their production and the subsequence raise of their price. Other problems caused by the massive use of these resources are of environmental nature: high emissions of greenhouse gas are generated by combustion of fossil fuels and the synthetic polymers that take 100 years or more to degrade, accumulate on landfills.

Motivated by these problems, many efforts have been done to develop production process of bio-based plastics and biodegradable polymers derived from renewable resources. Polyhydroxyalkanoates (PHA) are bio-based plastics that are also biodegradable, biocompatible and have similar physicochemical properties to polypropylene (PP) and polyethylene (PE). Due to these characteristics, PHA are promising polymers for the replacement of PP and PE.

PHA are naturally synthesized and stored intracellularly as carbon and energy reserves by a large number of bacteria. Given these characteristics there was a great interest to develop and optimize strategies to produce, extract and manipulate these bioplastics. In the last years, the principal focus of PHA research is to develop alternative and sustainable production processes aiming to substantially decrease PHA production costs and on the impact of process on environment. Investigated strategies include the use of genetic/metabolic engineering of strains to increase productivity and/or facilitate PHA extraction, the use of low cost substrates (by-products or agro-industrial wastes) and the use of mixed microbial cultures (MMC). MMC are open systems that do not require sterile conditions, which is reflected in lower investment and operating costs, consequently decreasing global PHA production costs. Although a decrease in PHA production costs through the use of MMC is generally accepted (estimated because MMC PHA production is not yet implement at an industrial scale), such costs are still high in comparison to synthetic plastics which has limiting the replacement of the latter by PHA.

Thus and despite the knowledge already available about PHA production in mixed culture processes, further investigation is required in order to better understand the process bottlenecks and to design new and more competitive strategies to produce PHA by MMC.

The main objective of this thesis was to develop and apply computational system biology tools and models to MMC PHA producing processes where complex mixtures of substrates as carbon source (real feedstock or synthetic substrates with more than two carbon sources) are used as feedstock since there are no studies reported to date. The real feedstock used as case study in this project was sugar cane molasses, a by-product of the sugar refinery industry with very high sugar content (over 50% dry weight). The models already available for this process using single and dual volatile fatty acids (VFA) as substrates were used as starting point to this work.

The major goals of this thesis can be pointed out as follows:

(I) to develop a metabolic network able to describe the PHA producing process using complex mixtures of VFA as feedstock;

(II) to develop a mathematical model and apply it to a real feedstock (sugar cane molasses) in order to understand the impact of sludge enrichment strategy and substrate complexity on the PHA metabolism;

(III) to extend the previous mathematical model (II) into a segregated form, i.e., a model able to discriminate populations within the MMC that can help to understand the metabolism of individual populations and how it affects PHA accumulation;

(IV) to extend the previous mathematical model (II) into a dynamic framework able to describe over batch time the uptake of complex mixtures of VFA and respective conversion into PHA.

1.2. Thesis Outline

The present Thesis is divided into eight chapters describing the work performed over the PhD project. Each chapter is structured as follows: first, a brief introduction where the respective state-of-the-art is reviewed; the main methodology used; presentation and discussion of the results is made and finally the main conclusions reached are highlighted. Three of these chapters (Chapter 5, 6 and 7) were published as scientific articles in peer reviewed international journals.

Briefly, each chapter includes the following contents:

- **Chapter 1** (present chapter) provides the motivation and the objectives of this Ph.D. thesis. It is also included the thesis outline with a brief summary of the contents of each chapter.
- **Chapter 2** provides the current state of the art in PHA production. Topics such as: I) the importance of PHA in terms of environmental and economical impact; II) the diversity of existing PHA and its properties as also several applications and III) the currently available strategies applied to PHA production by both pure cultures and MMC. The use of MMC brings several advantages over the use of pure cultures but also additional challenges principally due to population dynamics. And, in order to overcome this gap on MMC knowledge, the present work aims to develop new metabolic modelling strategies applied to these communities. In this chapter it is also made a briefly introduction to stoichiometric and constraint-based modelling that will be further complemented on the introduction of each chapters.
- The mathematical tools developed throughout this Ph.D. thesis were based in a MMC PHA-producing process using fermented sugar cane molasses as feedstock. This process comprises three stages: acidogenic fermentation of sugar cane, culture selection under aerobic dynamic feeding (ADF) regimen in a sequencing batch reactor (SBR) and maximization of PHA production on fed-batch using a pulse wise feeding strategy. In **Chapter 3**, the details of experimental set-up, analytical techniques and operating conditions of each stage are described. The experimental data (measured fluxes) of the PHA-producing batch experiments and the calculations of the kinetic and stoichiometric parameters for the 3rd stage of the process (PHA production) are also presented, since the mathematical techniques developed over this thesis were only applied to this stage.
- Before the development of the mathematical tools, a metabolic network able to represent the studied process has to be defined. The existing metabolic networks were only defined for simpler process where synthetic substrates were used and could not be applied to our system. Then, **Chapter 4** describes the development of a more complex metabolic network that could be applied to a MMC PHA-producing process using real substrates. The metabolic network developed was essentially an extension of a previous one (Dias et al., 2008). The material balances and theoretical yields were also defined and presented in this chapter.

- In **Chapter 5**, the metabolic network developed in Chapter 4 was validated using part of experimental data presented in Chapter 3. Metabolic flux analysis (MFA) and flux balance analysis (FBA) were implemented at different stages of culture enrichment in order to assess the impact of MMC enrichment strategy and substrate complexity on PHA metabolism. The metabolic model was also used to predict PHA production fluxes from substrate uptake rates.

- Structure-function studies in MMC can be performed using fluorescence in situ hybridization (FISH) combined with microautoradiography (MAR)-FISH. These techniques were applied to the same process studied through this thesis and it was able to identify the populations present in MMC as also their substrate specific consumption (Albuquerque et al., 2013). Using this information along with the experimental data of Chapter 3 and the metabolic network (Chapter 4), a segregated flux balance analysis method was developed to calculate metabolic flux distributions of individual populations present in MMC. **In Chapter 6** it is described how this method was developed and the results of its application to the MMC PHA producing process from fermented sugar cane molasses. It was also investigated the predictability of the method on this highly dynamic communities.

- **Chapter 7** describes the development of a dynamic metabolic model aiming to describe PHA production from mixtures of VFA along batch time. This dynamic model was based on the metabolic model developed in Chapter 5. The dynamic ability of this model was provided by the introduction of a cybernetic variable that represents VFA uptake regulation. The model was also calibrated with experimental data of several accumulation batch experiments using either fermented sugar cane molasses or a synthetic mixture of VFA. With this model, the kinetics of multiple VFA uptake could be evaluated as well as how the VFA uptake is affected along pulses and during the complete batch experiments by the regulation factor.

- Finally, **Chapter 8** provides the main conclusions achieved in this Ph.D. dissertation. It is also pointed some questions that arose subsequently to the present work and suggestions to address and to study them.

2 *State of the art*

2.1. Polyhydroxyalcanoates (PHA)

PHA are biopolymers used as intracellular carbon and energy storage by a wide variety of bacteria that have similar properties to several petroleum-derived plastics, such as polypropylene (PP) and polyethylene (PE). However, PHA are superior to them in many aspects including biocompatibility, biodegradability and the ability to being produced from renewable sources.

2.1.1. Economical and environmental importance of PHA

Plastics are seen as a 21st century material able to support the innovative developments required by a society constantly evolving. These materials cover a wide range of applications and enable to improve life quality and also a more efficient use of resources. In 2011, the global production of plastics reached 280 million tonnes, being PE (29%), PP (19%) and polyvinylchloride (PVC) (11%) the polymers with higher penetration in the market (Plastics – the Facts 2012; An analysis of European plastics production, demand and waste data for 2011). PE, PP and PVC are all synthetic polymers derived from fossil fuels taking many years (100 years or more) to decompose in the environment, which raise two questions: I) the finiteness of petroleum reserves and II) the accumulation of plastic in landfills representing a real environmental problem.

To solve these two questions the alternative material should be bio-based and could undergo composting/biodegradation. The use of raw materials such as sugar cane, corn and others bio-based feedstocks able the production of polymers which could be biologically degradable producing only water, carbon dioxide and inorganic compounds without toxic residues (Siracusa et al., 2008). A biopolymer that fulfils these both features, like polyhydroxyalkanoates (PHA), has a closed loop life cycle (Figure 2.1).

Currently, PHA is not a cost effective alternative to synthetic polymers due to high production costs. This biopolymer is only used in some niche applications where specific properties of the polymer are desired and the cost is not the most important factor, such as on medical devices. Development of more economic and efficient strategies has been intensified in last few years.

Life cycle assessment (LCA) studies were also conducted in order to evaluate the real impact of PHA and its overall production process on environment and can also give clues for process optimization. There are LCA studies for PHA production both by pure cultures (Harding et al., 2007) and MMC (Gurieff and Lant, 2007). In both cases, PHA production is advantageous over PP and PE production concerning to the global warming and energy requirements. Additionally, PHA production by MMC presents benefits regarding to the production process. Nevertheless,

the authors underline that the optimization of the later process is required to be more attractive in both financial and environmental aspects (Gurieff and Lant, 2007).

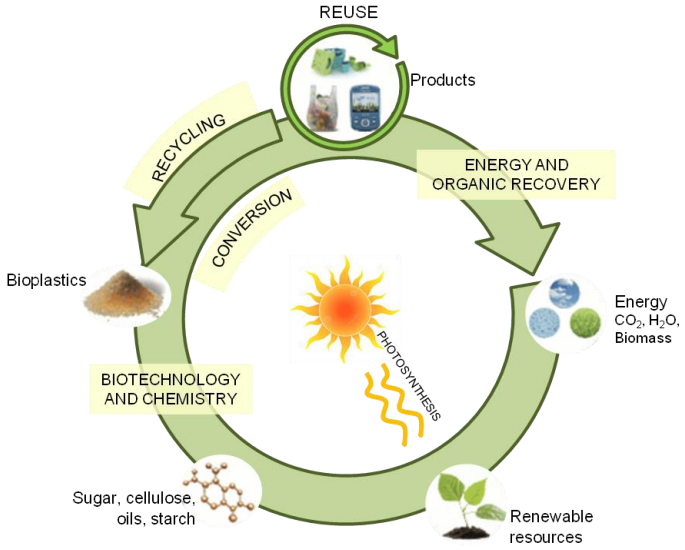


Figure 2.1: Bioplastics closed loop life cycle (adapted from www.european-bioplastics.org).

In this sense, further investigation on MMC PHA production process is mandatory to better understand all intervening points and to derive new process optimization strategies.

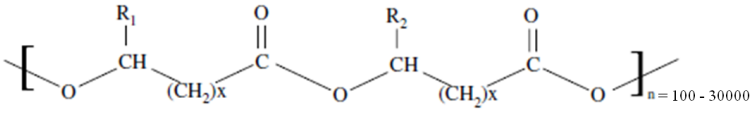
2.1.2. PHA structure, properties and applications

PHA belongs to a family of linear polymers constituted by hydroxyalkanoic acids. Their general structure and most common types are shown in Figure 2.2. All of them are piezoelectric and optically active (having only the (R)-configuration). They are also hydrophobic, insoluble to water, inert and indefinitely stable in air. They could also have thermoplastic and/or elastomeric properties (Laycock et al., 2012). However, there are other physical and chemical characteristics of PHA (melting and glass transition temperatures and level of crystallinity) highly dependent of the monomers presents in their composition. Depending on the number of carbon atoms in the monomers, PHA can be divided into two groups: short-chain-length (scl)-PHA (monomers with 3-5 carbon atoms) and medium-chain-length (mcl)-PHA (monomers with 6-14 carbon atoms).

The most common and studied PHA is poly(3-hydroxybutyrate) (P3HB) followed by poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)).

The average properties of homopolymer P3HB are glass transition temperature (T_g) ≈ 5ºC (determined by differential scanning calorimetry (DSC)) or 12ºC (determined by dynamic

mechanical thermal analysis (DMTA)); melting temperature (T_m) ≈ 176ºC; tensile modulus 2.9 GPa; tensile strength 37 MPa and maximum elongation to break ~ 4% (Laycock et al., 2012). These properties reflect in a high crystallinity and in a stiff and brittle material which are not the better ones in terms of processability and cause a disadvantage to use this polymer in a number of applications. Several studies were then made to surpass this barrier and most common solution reported to improve polymer mechanical properties is the use of copolymers such as P(3HB-co-3HV) or mcl-PHA. The presence of HV monomers decreases the stiffness and brittleness of the material, increasing its flexibility, tensile strength and toughness when compared with P(3HB) (Albuquerque et al., 2011).



x	R_1	R_2	Name	Abbreviation
1	H	H	Poly(3-hydroxypropionate)	P3HP
	CH_3	H	Poly(3-hydroxybutyrate)	P3HB
	CH_2CH_3	H	Poly(3-hydroxyvalerate)	P3HV
	CH_3	CH_3	Poly(3-hydroxy-2-methylbutyrate)	P3H2MB
	CH_2CH_3	CH_3	Poly(3-hydroxy-2-methylvalerate)	P3H2MV
	$(CH_2)_2CH_3$	H	Poly(3-hydroxyhexanoate)	P3HHx
	$(CH_2)_3CH_3$	H	Poly(3-hydroxyoctanoate)	P3HO
2	H	H	Poly(4-hydroxybutyrate)	P4HB
	CH_3	H	Poly(4-hydroxyvalerate)	P4HV
3	H	H	Poly(5-hydroxybutyrate)	P5HB
	CH_3	H	Poly(5-hydroxyvalerate)	P5HV

Figure 2.2: General structure of polyhydroxyalkanoates and most common representatives (adapted from Philip et al. (2007)).

Most of mechanical properties studies were performed using PHA produced by pure cultures because they require a considerable amount of polymer. For mixed culture PHA the majority of the studies derived mechanical properties of PHA from their chemical composition, thermal properties and molecular weight because amounts of PHA produced by MMC is still limited (Albuquerque et al., 2010a; Bengtsson et al., 2010; Serafim et al., 2008b). Recently, the mechanical properties of PHB and P(HB-co-HV) produced by MMC were also evaluated (Arcos-hernández et al., 2013; Dobroth et al., 2011), but the idea about the properties of mixed culture PHA is a combination of those obtained for pure culture PHA still subsists.

For mixed culture PHA it was generally observed a higher T_m and lower enthalpy of melting than the one reported for PHA from pure culture (Bengtsson et al., 2010). Bengtsson and coworkers (2010) suggested that these differences could be due to the feeding strategies adopted on mixed culture PHA production and their effect on the formation of the micro-block copolymers. Albuquerque and coworkers (2011) also reported that the fraction of monomers other than 3HB is significantly higher in PHA produced by mixed cultures from mixtures of VFA than the ones reported for pure cultures.

Both results support that the complexity inherent to the mixed cultures affects the final polymer properties (Laycock et al., 2012). So, these authors stated the importance to demonstrate the quality of mixed culture PHA to consistently meet the standards required for use in commercial applications and the need to perform more studies on thermo-mechanical properties of mixed culture PHA.

PHA degradation can take place in diverse environments, under aerobic or anaerobic conditions (Volova et al., 2007) and the resulting products are water and carbon dioxide or water and methane, respectively (Jendrossek and Handrick, 2002). Both bacteria and fungi are able to efficiently degrade PHA and the specificity and activity of their extracellular depolymerases (enzymes able to hydrolyze PHA) influences PHA degradation as well as the environmental conditions (temperature, salinity, acidity of the medium, etc) (Volova et al., 2007). PHA can be also chemically digested at non-biologically active environment using hot alkaline solutions.

The wide variety of produced PHA reflects also a broad range of applications for these materials. At first, PHA were used to make daily used products such as shampoo bottles and packaging materials and, in 1990, appeared the first consumer product made of PHA. Since then, the use of these biopolymers is associated to areas such as industry, medicine and agriculture.

There are few companies producing PHA at industrial scale, namely Biomer, Metabolix and Tianan (China) - in collaboration with others. These biopolymers are currently used in a wide range of items in several areas, such as food packaging (Siracusa et al., 2008), food additives and medical and agricultural fields (Philip et al., 2007). PHA can be also blended with high or low molecular weight molecules or with inorganic phases (usually hydroxyapatite, bioactive glass, and glass–ceramic fillers or coatings) to enhance their properties (Philip et al., 2007).

The majority of the mentioned applications requires PHA with high quality and purity. However, mixed cultures PHA can have a high contribution for agricultural applications and others where the use of renewable feedstocks is an advantage.

2.2. PHA Production

More than 300 different microorganisms synthesize PHA as a natural way to store carbon and energy under stress conditions caused by limitation of a nutrient, electron donor or acceptor (Anderson and Dawes, 1990). Considering a production in large-scale, bacteria are the main candidates. Indeed, the occurrence of PHA in these organisms is known since 1920s, when PHB was identified by the first time inside bacteria (Lemoigne, 1926).

PHA are insoluble in water as such the polymers are accumulated in intracellular granules on the cell cytoplasm. Typically, in each cell, is observed 8 – 13 discrete granules with diameters ranging from 0.2 to 0.7 mm. Each granule is surrounded by a phospholipid monolayer with embedded proteins (Braunegg et al., 1998). Some of these proteins are the enzymes involved in PHA synthesis and degradation as well as phasins, proteins which are thought to be involved in the formation of the PHA granules (Pötter and Steinbüchel, 2005; Sudesh et al., 2000).

Cupriavidus necator (formelly called as *Ralstonia eutropha* or *Alcaligenes eutrophus)* is the most well studied PHA producing organism. The PHB metabolism involves three biosynthetic enzymes which converts acetyl-Coenzyme-A (acetyl-CoA) into PHB when a carbohydrate (e.g. glucose) is used as substrate. Acetyl-CoA is the key compound of PHB biosynthetic pathway. After glucose is converted into acetyl-CoA through glycolysis, the PHB biosynthetic pathway begins with 3-ketothiolase (PhaA) combining two molecules of acetyl-CoA to form acetoacetyl-CoA. Then, acetoacetyl-CoA reductase (PhaB) converts acetoacetyl-CoA into 3-hydroxybutyryl-CoA. Finally, PHB synthase (PhaC) polymerizes 3-hydroxybutyryl-CoA into PHB and coenzyme-A is released (Figure 2.3) (Sudesh et al., 2000). Since PHB is a carbon and energy reserve, PHB producing organisms also have an enzyme, PHA depolymerase (PhaZ), able to degrade the polymer and restore the HB monomers that could be further converted into acetyl-CoA to be used in other cellular pathways (Sudesh et al., 2000). This pathway is similar for the biosynthesis and degradation of other PHA and the specificity of the PHA synthase (PhaC) determines the type of PHA product, either scl-PHA or mcl-PHA (Rehm, 2003).

Other substrates such as volatile fatty acids (VFA), (i.e. acetic, propionic, butyric and valeric acids) can be used as substrates for PHA production. In this case, VFA are intracellularly activated to the corresponding acyl-CoA molecules. Acetyl-CoA can enter directly into PHB biosynthetic pathway. Propionyl-CoA can be decarboxilated into acetyl-coA and form PHB by combining two molecules of acetyl-CoA or form PHV by combining acetyl-CoA with propionyl-CoA molecules. Butyryl-CoA and valeryl-CoA can be directly converted into hydroxybutyryl-CoA and hydroxyvaleryl-CoA, which can subsequently be used for PHB and PHV synthesis.

Usually, when mcl-PHA are synthesized, long chain VFA is used as substrate. Long chain VFA are then converted to their corresponding CoA derivative and then enter in β-oxidation pathway (Figure 2.3). Different types of acyl-CoA molecules can be generated resulting in different mcl-PHA. At a lesser extent, other carbon sources also can be used as substrates for mcl-PHA synthesis. In carbohydrate case, hydroxyacyl-CoA (the PHA precursor) can be formed through *de novo* fatty acid biosynthesis and in alkanes case the PHA precursor results from the activation of the corresponding acyl-CoA molecule generated by the alkane oxidation (Figure 2.3).

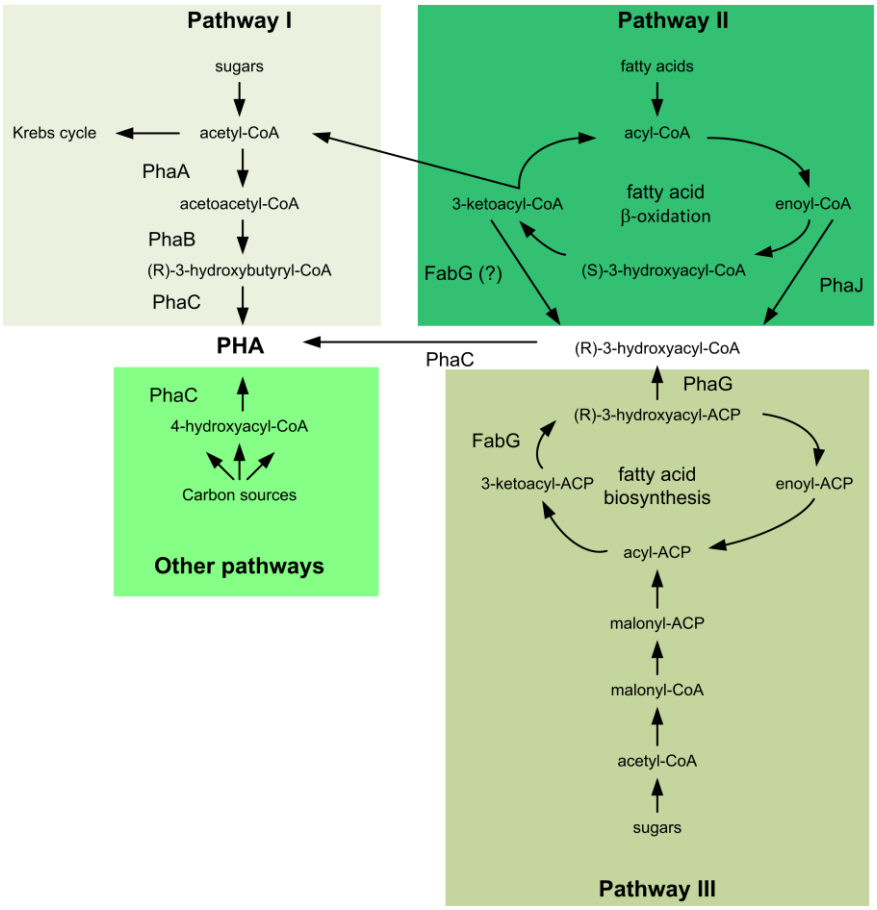


Figure 2.3: Several metabolic pathways able to produce monomers for PHA synthesis (Tsuge, 2002).

2.2.1. Current industrial PHA production

Since 1980's, many companies have devoted a considerable effort in research and development of strategies for PHA production by microorganisms at pilot and industrial scale. So far, all the industrial processes implemented use pure microbial cultures (wild or genetically modified strains). Usually, in these cultures, PHA production is performed by fermentation under batch or fed-batch mode. Firstly, the culture is fed with a growth medium to reach a high cell density

without a significantly accumulation of PHA. After the depletion of the growth medium, a growth-limiting medium is supplied to the culture in order to induce or maximize the production of PHA. The limitation of oxygen or a macroelement, such as ammonia or phosphate, on the presence of carbon excess is the most used strategy to stimulate PHA production. When the biomass reaches their maximum PHA content, the cells are recovered and disrupted in order to extract the intracellular PHA.

Currently there are about 14 companies producing PHA. Despite the wide variety of known PHA only a few are produced on an industrial scale: PHB, P(HB-co-HV), P(HB-co-HHX) and, in lesser cases, P(HB-co-4HB) and mcl-PHA (Chen, 2009). The most commonly used organisms are *R. eutropha*, *Alcaligenes latus*, *Aeromonas hydrophila*, *Pseudomonas oleovorans* and *P. putida* as natural PHA producers and genetic manipulated *Eschericia coli* due to its convenience for genetic manipulation, fast growth, high final cell density and ability to utilize inexpensive carbon sources (Table 2.1).

A great improvement in PHA production was already achieved and the results obtained so far are promising. However the production cost of these biopolymers is still too high to compete with synthetic plastics (average of €3/kg for polyhydrobutyrate (PHB) against €1/kg for petroleum-based plastic) (Chanprateep, 2010).

The optimization and effective production of PHA depends on a number of factors like final cell density, bacterial growth rate, PHA content in cell dry weight, total time until reach high final cell density, substrate and operating costs and suitable and economic method to extract and purify the polymer. The high price of PHA may result mainly from the high costs of substrates and of the equipment and energy required for aseptic operation as well as the substantial number of unitary operations, chemicals and energy demand required in downstream processing (Choi and Lee, 1999).

Table 2.1: Bacterial strains commonly used for pilot and large scale PHA production (adapted from Chen, 2009).

Strain	DNA manipulation	PHA type	C-source	Company
Ralstonia eutropha	No	PHB	Glucose	Tianjin North. Food, China
Alcaligenes latus	No	PHB	Glucose or sucrose	Chemie Linz, btF, Austria. Biomers, Germany
Escherichia coli	*phb* CAB + *vgb*	PHB	Glucose	Jiang Su Nan Tian, China
Ralstonia eutropha	No	P(HB-co-HV)	Glucose + propionate	ICI, UK. Zhejiang Tian Na, China
Ralstonia eutropha	No	P(3HB-co-4HB)	Glucose + 1,4-BD	Metabolix, USA. Tiajin Green Biosci. China
Escherichia coli	*phb* CAB			
Ralstonia eutropha	*pha* C_{Ac}	P(HB-co-HHX)	Fatty acids	P&G, Kaneka, Japan
Aeromonas hydrophila	No	P(HB-co-HHX)	Lauric acid	P&G, Jiangmen Biotech Ctr, China
Aeromonas hydrophila	*phb* AB + *vgb*	P(HB-co-HHX)	Lauric acid	Shandong Lukang,
Pseudomonas putida	No	mcl PHA	Fatty acids	ETH, Switzerland
P. oleovorans				
Bacillus spp.	No	PHB	Sucrose	Biocycles, Brazil

vgb : gene enconding *Vitreoscilla* hemoglobin; *phb* CAB: PHB synthesis genes enconding β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase; 1,4-BD: 1,4-butanediol; *pha* C_{Ac} : PHA synthase gene *pha* C from *Aeromonas caviae* ; *phb* AB encodes β-ketothiolase and acetoacetyl-CoA reductase.

2.2.2. Low cost PHA production strategies – Mixed Microbial Cultures (MMC)

The use of mixed microbial cultures and waste materials as feedstocks are reported as two approaches able to decrease PHA production costs by avoiding, respectively, sterilization and substrates costs (Serafim et al., 2008a). Even if the price of PHA becomes slightly higher than that of synthetic plastics, the biodegradability of PHA may compensate the environmental problems caused by the synthetic polymers.

In order to implement an efficient process able to produce PHA at large scale, using one or both approaches, several studies have been performed. This evidence is demonstrated by the increased numbers of articles about PHA and MMC published in the last decade (analysis made in ISI Web of Knowledge).

Mixed microbial cultures are usually a consortium of microbial populations in open biological systems whose composition depends on the substrates and operational conditions imposed to the bioreactor. One of the most known MMC is activated sludge present in biological wastewater treatment plants (WWTP). Curiously, it was in one of these systems (enhanced biological phosphorous removal; EBPR) that the storage of PHA was observed for the first time in a MMC (Wallen and Rohwedder, 1974). This process is operated by alternating anaerobic/aerobic cycles and the microorganisms responsible for PHA storage are polyphosphate-accumulating organisms (PAOs) and their competitors, glycogen-accumulating organisms (GAOs). Indeed,

PHA plays an important role in both their metabolisms. Under anaerobic conditions, both PAOs and GAOs store PHA from external carbon sources and internal glycogen previously stored. Next, on aerobic phase, PHA is consumed for cell growth, maintenance and glycogen pool replenishment.

PHA storing was also observed in fully aerobic WWTP where selectors for bulking control were introduced. In this process, the sludge is submitted to alternate periods of excess (in the selector reactor) and lack (in the main reactor) of carbon (Majone et al., 1996). Currently, this process is known as ‘‘aerobic dynamic feeding’’ (ADF) or ‘‘feast and famine’’ (FF). During the feast phase external substrate is consumed for simultaneous growth and PHA storage and when substrate is exhausted (famine phase) the accumulated PHA can be used as carbon and energy source. In this way, the microorganisms obtain enough energy for storage, growth and substrate uptake from oxidizing part of the substrate.

In both strategies, anaerobic/aerobic cycles and ADF, PHA storage occurs when growth is prevented, thus the anaerobic/aerobic process also can be classified as FF regime. In the ADF process, electron donor and acceptor are present during the substrate uptake and then which prevents growth is an internal limitation (insufficient intracellular components such as enzymes and/or RNA). In the anaerobic/aerobic process, it is the absence of an electron acceptor (oxygen, nitrate) that limits growth (external limitation) and promotes PHA storage.

Over anaerobic/aerobic process the ADF process has the advantage of being independent of polyphosphate and glycogen synthesis avoiding the tendency to accumulate other storage compounds. Then the majority of the studies on MMC PHA use fully aerobic FF regimen.

Mixed microbial cultures PHA production processes usually use two or three step strategy depending on the origin of the feedstock, synthetic or waste-based respectively (reviewed by Dias et al., 2006 and Serafim et al., 2008a)(Figure 2.4). The majority of the waste-based feedstocks are carbohydrate-rich mixtures of compounds (sugar cane and beet molasses, cheese whey, olive oil mill among others) and they are rarely used directly for PHA production since microorganisms tend to storage glycogen rather than PHA when they use those carbon sources (Carta et al., 2001). Since the use of waste-based feedstocks may decrease PHA production costs, a way to overcome this problem is to add a previous step in PHA production process in order to convert the carbohydrate-rich substrates in a more readily carbon substrates to store PHA like organic acids (step 1 in Figure 2.4). The other two steps (2 and 3 in Figure 2.4) are: the culture selection (2) where the MMC is enriched in PHA storing organisms under dynamic feeding conditions and the accumulation step (3) where PHA production is maximized.

Both steps, 2 and 3, can use the feedstock previously prepared in step 1. The separation of the culture selection and PHA production is an important point because it allows to use different operating conditions as also as independent strategies for each step.

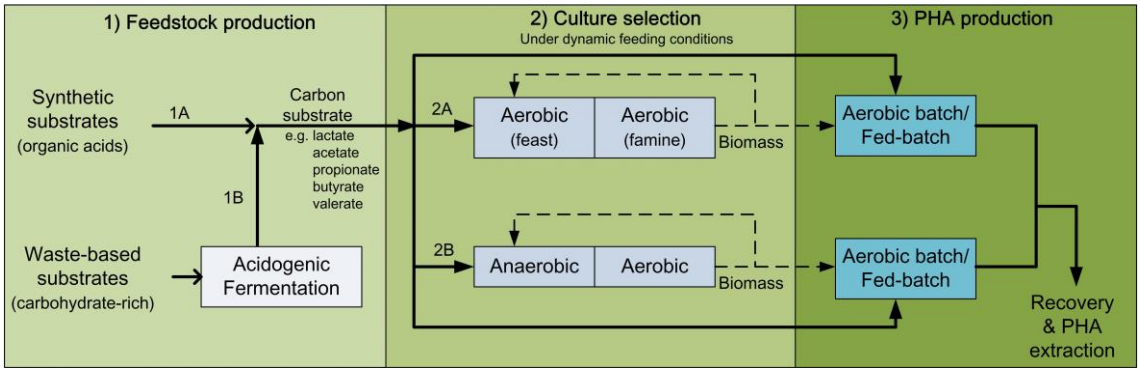


Figure 2.4: Three-step PHA production process by MMC using either synthetic (1A) or waste-based substrates (1B) and performing culture selection using Aerobic/Aerobic (2A) or Anaerobic/Aerobic (2B) dynamic feeding strategies. PHA production (step 3) is carried out in batch/fed-batch mode using the cultures enriched in step 2 and the feedstock produced in step 1 (Serafim et al., 2008a).

Sequencing batch reactor (SBR) is the most frequent configuration used in MMC PHA selection. This configuration allows to perform a full FF cycle where the same sludge retention time (SRT) and the organic loading rate (OLR) can be maintained independently of the length of each phase. However, as alternative, continuous reactors were also used in PHA production (Albuquerque et al., 2010a; Bengtsson et al., 2008). Albuquerque and coworkers (2010a) compared the performance of culture selection for both configurations (SBR and continuous reactor). They obtained similar results in both configurations for PHA content, polymer yield on substrate and specific productivity which supports the possibility of using the existing facilities of WWTP for PHA production from industrial or municipal effluents.

An important issue for the optimal performance of the entire PHA production process is the efficiency of the culture selection step. In order to optimize this step there are two main goals that must be considered: I) enrichment of an activated sludge in organisms with high and stable PHA storage capacity and II) performing the enrichment at the highest possible rate and concentration of biomass (i.e. biomass volumetric productivity).

So far, the first goal has already been achieved. There are studies of MMC enrichments reporting 80-90% of PHA-storing organisms in the overall biomass, showing high PHA content and specific PHA productivity in the final production step (Albuquerque et al., 2010b; Jiang et al., 2011b; Johnson et al., 2009a).

Although the intense research on MMC PHA production, the second goal is still a challenge. The ideal situation is to achieve a selected culture with high growth rate and high storage capacity. This culture would enable to operate the selection reactor at high cell concentration to support the feeding of a more concentrated inoculum to the subsequent production step. The main underlying bottleneck is to identify the optimal conditions to operate this reactor during the FF regimen. Since the FF regime causes an internal growth limitation to microorganisms (which promotes PHA storage and confers them a competitive advantage over the organisms without this capacity) a compromise has to be attained between polymer storage and growth response in order to optimize the operational conditions of the selection reactor.

Table 2.2: Common values of operating parameters used for PHA accumulating culture selection in mixed culture processes (Reis et al., 2011).

Operating parameter	Range
Related to reactor operating parameters	
Sludge retention time (SRT)	1 - 20 day
Hydraulic retention time (HRT)	1 - 3 day
Length of SBR cycle	2 - 12 h
pH	7 - 9.5
T	20 - 30ºC
Operation mode	SBR or two continuous reactors sequentially disposed
Related to feedstock	
Organic loading rate (OLR)	1.8 - 31.25 g-COD l^{-1} day^{-1}
Substrate concentration	0.9 - 31.25 g-COD l^{-1} day^{-1}
C/N ratio	9 - 120 g-C gN^{-1}
Resultant from both feeding and reactor parameters	
F/F ratio	0.1 - 1.15

There are many studies reporting the factors that may affect the selective pressure imposed on culture selection during the feast phase under different reactor operating conditions (reviewed by Reis et al., 2011). Among others, SRT, hydraulic retention time (HRT), pH, temperature, cycle length, number of cycles per SRT, OLR, influent substrate and nutrient concentration and the nature of the feedstock are considered to be the most relevant. Table 2.2 lists the parameters and respective range of operation that were investigated so far. These parameters have a consequent impact in feast to famine length ratio (F/F ratio) and kinetic effects associated with carbon substrate concentration (limitation/inhibition) (Reis et al., 2011).

The optimal operational conditions for the culture selection reactor were then identified by several authors (Albuquerque et al., 2010b; Chua et al., 2003; Dionisi et al., 2005b; Johnson et

al., 2009a; Serafim et al., 2004; Villano et al., 2010). A summary of these results is presented in Table 2.3.

Table 2.3: Summary of better operating parameters to obtain an enriched PHA-storing culture.

Operating parameter	Impact	Better for select PHA-storing organisms	References
Sludge retention time (SRT)	Reflects inversely on specific growth rate	inconclusive because high enriched PHA cultures were selected under SRT = 10 d or SRT = 1 d.	Albuquerque et al. 2010b (SRT=10d) Johnson et al. 2009 (SRT=1d)
F/F ratio	Varied according OLR and/or influent substrate concentration	Lower F/F ratio (±0.2) promotes high storage response.	Albuquerque et al. 2010b Dionisi et al. 2005b
C/N ratio	Allow a carbon limited FF cycle where growth could occur from PHA stored (famine phase) or a nutrient limited FF cycle where growth only occur in feast phase	Carbon limited FF cycle seems to be better for the subsequent production phase (cultures became more insensitive to the presence of nutrients which is very important when feedstocks with nitrogen sources are used).	Serafim et al. 2004
pH and T	Influence microbial culture selection	Difficult to assess on selection phase but generally pH around 7 is used. In production phase better results are obtained with similar pH used on culture selection.	Chua et al. 2003 Vilano et al. 2010

Similarly with the culture selection step, there are many studies reporting the optimization of the PHA production step by varying the following operational condition: feeding regimen, C/N ratio, OLR, pH and temperature as also the feedstock itself (type and amount of VFA) (Albuquerque et al., 2011; Dionisi et al., 2005b; Johnson et al., 2010a, 2010b; Krishna and Van Loosdrecht, 1999; Serafim et al., 2004; Villano et al., 2010). The efficiency of the culture selection step plays an important role in the performance of the subsequent PHA production step and on its optimization. The performance of the accumulation reactor was evaluated according to the maximum PHA cell content, PHA yield on substrate and PHA specific storage rate. PHA cell content could be considered the most important since it has a direct impact on the downstream costs because the higher PHA content the lower its extraction and purification costs.

According to the results so far reported, it is difficult to define an optimal strategy to adopt in the selection step. In these studies different cultures with high PHA cell content (>74%) were selected using different operating conditions (Albuquerque et al., 2010b, 2011; Jiang et al., 2011; Johnson et al., 2009; Serafim et al., 2004). For the accumulation step, a common strategy used in all studies was the use of nutrient-limiting conditions during this entire step. Under these

conditions, the uptake of carbon is mainly driven for PHA storage until it reaches a saturation level inside the cell. On the other hand, when nutrients are all in excess during the accumulation step, the growth response tends to increase (which may increase PHA volumetric productivity) but the storage response progressively decreases along with the PHA storage capacity (Reis et al., 2011).

The highest PHA content reported so far for a MMC fed with a waste-based feedstock (in this case fermented sugar cane molasses) was 74.6% (gPHA $gVSS^{-1}$)(Albuquerque et al., 2010b). The adopted strategy was a pulse-wise feeding under nutrient-limiting conditions. For MMC using a single synthetic substrate (in this case lactate) this value was slightly higher, 92% (Jiang et al., 2011b). While in the latter case a homopolymer, PHB, was produced, on the case of Albuquerque et al. a copolymer (PHB-co-HV) was formed. So, the former strategy is more favourable to develop a process to produce PHA with a wide range of different characteristics.

Under a non-nutrient limiting strategy, the highest PHA content reported for a MMC in the accumulation step using fermented olive mill effluents was 54% (gPHA $gVSS^{-1}$) (Dionisi et al., 2005a). However, the culture selection was performed using synthetic substrates. Although this value is lower than the previous ones, this strategy should be considered for feedstocks containing nitrogen sources in order to increase the range of feedstocks which can be used for PHA production.

The use of MMC for PHA production still has lower performance than pure cultures in terms of final PHA content and PHA volumetric productivity; however for both these parameters, the results are becoming closer. Also, the results achieved with waste-based feedstocks are closer to the ones using synthetic substrates, encouraging further development.

2.3. Challenges in understanding mixed microbial cultures

The majority of the microorganisms present in natural environmental niches, as also in MMC, cannot be cultured with available cultivation technologies (Abulencia et al., 2010; Streit et al., 2004); therefore the study of these communities brings additional challenges over the study of pure cultures. New and adapted strategies have to be adopted in order to overcome this difficulty because these cultures present some advantages such as adaptability for example

There are MMC enriched into a monoculture, with high PHA storage capacity (Jiang et al., 2011b; Johnson et al., 2009a), and then some pure cultures technologies could be applied. However MMC with a diversified population will be advantageous over a monoculture when

considering the use of waste-based feedstocks since the first ones could easily adapt to fluctuations in operating parameters and feedstock composition.

Some new molecular and –omics techniques and tools can be applied to complex systems such as RNA profiling, metabolomics and proteomics; however, for detailed physiological characterization, it is still obligatory the establishment of pure cultures (Sabra et al., 2010). In order to overcome this gap on MMC knowledge, metabolic modelling strategies have been developed and applied to these communities.

2.3.1. Stoichiometric and constraint-based modelling

The number of reactions occurring within one cell and among a population is very large and complex and normally only part of the underlying mechanisms is currently known. Consequently, the fully kinetic description of the involved reactions and regulation mechanisms is out of reach. In order to minimize this gap, the development of new metabolic modelling strategies, under systems biology approach, is mandatory to address some important issues such as cell and population behaviour under different environment conditions.

During their life cycle, cells have to abide several constraints of different kinds that affect their intracellular processes: stoichiometric, physicochemical (e.g. conservation of mass and energy, thermodynamic, maximal reaction rates); topological (e.g. macromolecular interactions, diffusion of large molecules); environmental (e.g. pH, temperature, availability of carbon and nutrients sources) and regulation constraints.

Within the scope of MMC, most of the metabolic modelling developed so far is based on stoichiometric and constraint-based modelling (reviewed by Dias et al., 2006). In stoichiometric modelling, the reaction stoichiometry of the metabolic network is the starting point. In this approach, the dynamic intracellular behaviour is disregarded, based on the assumption of the pseudo steady-state for internal metabolites (Stephanopoulos et al., 1998). The major advantage of using this modelling approach is that the cell metabolism can be exploited without kinetic information about intracellular metabolites.

Apart of stoichiometric ones, additional constraints can be included on metabolic modelling. Here, the major challenge is to enumerate, understand and apply these constraints into computational framework and to convert the available information about the metabolism into mathematical models. The success of this strategy will allow to define more accurately cellular capabilities(Covert et al., 2003).

Mathematically, stoichiometric and constraint-based modelling can be translated into a set of equations as represented in Figure 2.5. The metabolites and reactions defined in the metabolic network, as well as their stoichiometry, can be represented by a stoichiometric matrix, in which rows corresponds to **m** metabolites and columns to **n** reactions. This matrix is of major importance since it stands for the translation of biological knowledge into mathematical terms.

Then, the stoichiometric matrix allows to represent the mass balances of intracellular metabolites by a set of ordinary differential equations:

$$\frac{dc}{dt} = S \cdot v - \mu \cdot c \tag{2.1}$$

where $c = (c_1, c_2, ..., c_m)$ is the vector of intracellular metabolite concentration, $v = (v_1, v_2, ..., v_n)$ the flux vector, and μ the specific growth rate of cells. This dynamic mass balance equation describes the evolution over time of the metabolite concentration, c_m. According with this equation, data about stoichiometry (S), biomass growth (μ) and intracellular reaction fluxes (v) is required in order to model the dynamic evolution of intracellular metabolites. As mentioned earlier, for the majority of the biological systems some of this data (e.g. intracellular reaction fluxes) are very complex, difficult to measure and understand. Thus, in a way of simplicity, pseudo steady-state for internal metabolites can be assumed, as the intracellular dynamics is much faster than extracellular dynamics and this dynamic can be disregarded. The dilution term $\mu \cdot c$, that affects the metabolites, is normally much smaller than the fluxes and can also be disregarded (Stephanopoulos et al., 1998). Despite the negligence of these two terms, the dynamic of extracellular processes (e.g. substrate uptake and product formation) is still considered and then the dynamics of the macroscopic processes is not lost. By considering the previously described assumptions, the mass balances of each metabolite, also called as general equation, can be expressed as a homogeneous system of linear equations:

$$0 = S \cdot v \tag{2.2}$$

This equation contains all feasible solutions for flux distribution, v, corresponding to the metabolic network defined by S. These solutions represent the phenotypes that cells can adopt according to the different environmental or physiological conditions. Considering that S is a full row rank, then **m** is the number of independent equations. Usually, in a metabolic network, the number of reactions, **n**, is higher than the number of metabolites, **m**, and the resulting system is undetermined with **n-m** degrees of freedom.

The identification of additional constraints is critical to reduce the solution space of system 2.2 and to facilitate the identification of a unique or reduced number of feasible flux distribution that

match with the experimental data, i.e. measured fluxes. These more realistic solutions will enable to analyse, interpret and predict the phenotypes of the cells in the context of a population and external conditions (Covert et al., 2003).

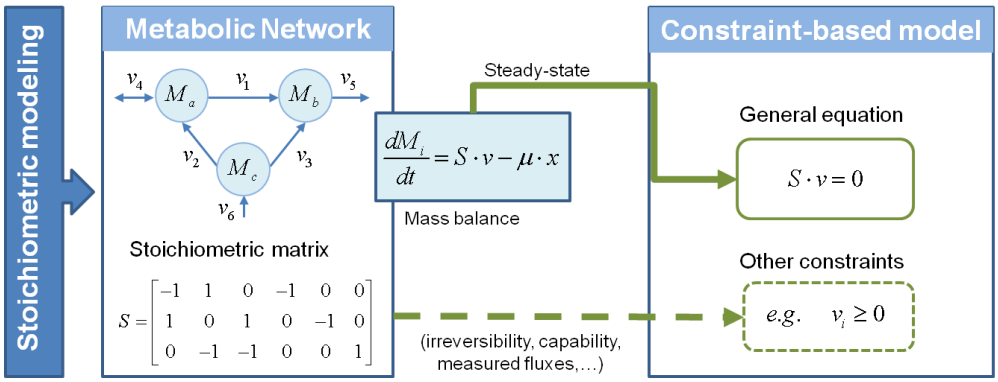


Figure 2.5: Principles of stoichiometric and constraint-based modelling. The metabolic network, and the stoichiometric information embedded on it, can be translated into mathematical terms and represented by a matrix. Using this matrix, the mass balances for each metabolite can be mathematically represented by a set of ordinary differential equations. Assuming pseudosteady state for intracellular metabolites, the mass balances can be described by a homogeneous system of linear equations (called as general equation). This equation constraint the flux distributions that can be achieved by the metabolic network, but it does not predict the actual distribution. Finally, additional constraints, such as irreversibility or capacity constraints, can be incorporated in order to determine the flux distributions that can be achieved by a cell under given conditions. (Llaneras and Picó, 2008)

In the context of MMC, the major computational challenge, and perhaps its success, is to develop adequate constraint-based models that fit with such complex systems. Over last years, metabolic modelling have been applied to MMC systems, namely on PHA production processes. The current state of the art on this subject will be explored in Chapter 4, as well as the development of a metabolic network applied to such process using complex mixtures of fermented compounds as substrate.

3 Experimental data

Within this thesis the mathematical tools developed were based in a MMC PHA-producing process using fermented sugar cane molasses as feedstock. To convert sugar cane molasses into PHA a three stage process is needed. Firstly, since PHA-producing organisms cannot accumulate PHA from sugar-rich substrates (instead they produce glycogen), the sugar cane molasses have to be fermented in order to produce volatile fatty acids (VFA). Secondly, after being clarified, the fermented sugar cane molasses and a mineral nutrients solution are fed to a sequencing batch reactor (SBR) where the selection of PHA-producing organisms takes place. This selection was achieved by imposing alternating periods of excess and absence of carbon source (feast and famine regimen) to the SBR. In order to maximize PHA production, a fed-batch reactor is inoculated with SBR sludge enriched in PHA-producing organisms and fed with clarified fermented sugar cane molasses following a pulse-wise feeding strategy combined with ammonia limiting conditions. This 3-stage process were previously developed by Albuquerque and coworkers (2007; 2010b).

In this chapter, the details of experimental set-up, analytical techniques and operating conditions of each stage were described. The kinetic and stoichiometric parameters for the 3rd stage of the process (PHA production) were calculated. Since mathematical techniques developed over this thesis were only applied to the 3rd stage only the experimental data (measured fluxes) of the PHA-producing batch experiments are presented here.

3.1. Experimental setup

The MMC PHA-producing process used as case study in this thesis was developed by Albuquerque and coworkers (2007; 2010b).

The experimental setup for the PHA production process consisted of a three bench-scale reactor systems (Figure 3.1) comprising a molasses acidogenic fermentation (stage 1), a PHA-accumulating culture (stage 2) and PHA production fermentation (stage 3). The stage 1 was operated in a continuous stirred tank reactor (CSTR) under anaerobic conditions. For the stage 2 a sequencing batch reactor (SBR) subjected to feast and famine conditions was used; and stage 3 was carried out in a batch reactor inoculated with sludge from the culture enrichment SBR and fed with clarified fermented molasses produced in stage 1.

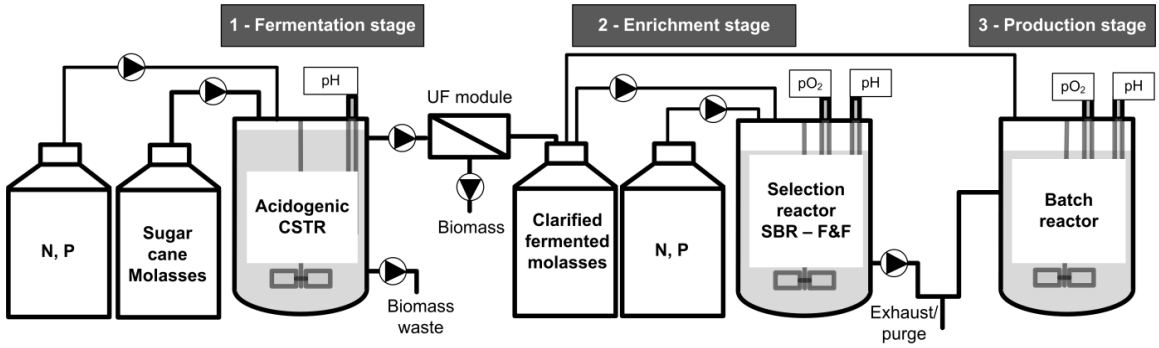


Figure 3.1: Experimental setup of MMC-PHA production from sugar cane molasses. (adaptation from (Albuquerque et al., 2010b)).

3.1.1. Continuous stirred tank reactor (CSTR)

A CSTR with a working volume of 1140 mL was operated under anaerobic conditions for a period of over three years. The operating conditions of the reactor were: pH 6, temperature of 30ºC and hydraulic retention time (HRT) of 10h (Albuquerque et al., 2007). The influent substrate was kept at 4ºC and resulted from the combination of two solutions: a molasses solution (prepared by diluting sugar cane molasses with tap water 1:2 with a final concentration of 220 g/L of sugars) and a mineral nutrients solution containing ammonia and phosphate at concentrations according to a C/N/P ratio of 100:3:1 C-mol:N-mol:P-mol inside the reactor. The final influent substrate contained 10 g/L of sugars.

The sugars contained in the molasses (75% of the soluble total organic carbon (TOC) of molasses prior to fermentation) were converted (more than 99%) to VFA according to a yield of about 0.70 C-mol VFA/ C-mol sugars. The products resulted from the fermentation were acetate,

propionate, butyrate and valerate that account for 75% of the soluble TOC of fermented molasses.

The effluent from the CSTR was withdrawn by overflow and further microfiltrated through a hollow fiber membrane module in order to remove biomass. The clarified effluent was kept at 4ºC prior to its use as the feedstock for 2nd (culture enrichment) and 3rd (PHA production) stages of the process.

3.1.2. Sequencing batch reactor (SBR)

Culture selection was carried out in a sequencing batch reactor (SBR) with a working volume of 800 mL. The SBR was inoculated with a PHA-accumulating mixed culture previously acclimatized to the fermented molasses feedstock (Albuquerque et al., 2007) and operated under the conditions compiled in Table 3.1. SBR was operated at cycles of 12h divided into the following phases: fill (5 min); aerobiosis (feast and famine) (11 h); settling (45 min); and supernatant withdraw (10 min). Following the settling phase, the exhaust supernatant was withdrawn (400 mL/cycle). The sludge retention time (SRT) was kept constant at 10 days by imposing a purge of mixed liquor at the end of the reaction phase.

Table 3.1: Operating conditions for SBR at different enrichment periods.

	Operating Conditions				Influent Concentrations			
Enrichment period (days)	OLR (C-mmolVFA/L.d)	HRT (days)	SRT (days)	Cycle lenght (h)	VFA (C-mmol/L)	NH_4 (N-mmol/L)	PO_4 (P-mmol/L)	C/N/P (C-mol/N-mol/P-mol)
0 - 70	60	1	10	12	30	2.5	0.32	100/8/1
71 - 208	120				60	5	0.68	
209 - 900	90				45	3.75	0.48	

The feed solution for the SBR contains a mixture of the effluent from the acidogenic fermentation (carbon source) with a mineral nutrient solution (maintained in separated containers and fed simultaneously by two peristaltic pumps). The effluent containing about 180 C-mmol VFA/L was diluted with the mineral solution (ammonia and phosphates) according to the desired VFA concentration inside SBR (see Table 3.1) obeying always to the C/N/P ratio of 100/8/1. After dilution, the effluent pH was adjusted to 8 ± 0.05 and stored at 4ºC (see table 3.1). Allylthiourea was also added to this solution in order to inhibit nitrification.

Air was supplied to the reactor through a ceramic diffuser. Stirring was kept at 500 rpm. pH was not controlled. Pumping (fill and withdraw), aeration, and mixing were automatically controlled by a software program developed in our research lab. In addition, the software also

acquires pH and dissolved oxygen data online. The reactor was operated in a temperature-controlled room between 23 and 25°C.

3.1.3. PHA accumulation batch

Batch PHA accumulation experiments were performed at different periods of the culture enrichment in order to evaluate the capacity of culture to store the biopolymer. Some experiments were carried out in a 600 mL working volume reactor (reactor 1), while others were conducted in a 1100 mL working volume reactor (reactor 2, B.Braun Biostat A). In reactor 1, air was supplied by an air pump through a ceramic diffuser and in reactor 2, aeration was controlled by a mass gas flowmeter and kept at 400 mL/min. In reactor 1, stirring was kept at 400 rpm, while in reactor 2, stirring was kept at 300 rpm. In both reactors pH was monitored (but left uncontrolled and ranging between 8 and 9). Temperature in both reactors was kept between 23-25ºC.

In all experiments, reactors were inoculated with the SBR enriched sludge collected at the end of the SBR famine phase: 200-300 mL were used to inoculate reactor 1, while 400 – 500 mL were used to inoculate reactor 2. The reactors were then fed with either clarified fermented molasses or with a synthetic VFA mixture of acetate, propionate, butyrate and valerate mimicking clarified fermented molasses composition (designated as simulated feed). pH of the feeding solution in both solutions was adjusted to 8 prior to reactor feeding. In all batch experiments, a pulse wise feeding strategy (3 – 6 pulses of 60 – 100 C-mmol VFA/L) was adopted. Dissolved oxygen (pO_2) was used as an indicator of the VFA exhaustion: a sudden rise of pO_2 indicated that substrate was fully exhausted. In tests with fermented molasses a volume between 200 and 300 mL of exhausted medium was replaced by fermented molasses in the end of each pulse, after sludge settling process, which taken about 10 min. This volume was adjusted according to the desired initial concentration of VFA (between 60 and 100 C-mmol VFA/L). This procedure was required to cope with the diluted concentrations of VFA (approximately 180 C-mmol VFA/L) obtained from stage 1. In the case of the simulated feed, concentrated solutions of synthetic VFA (approximately 1000 C-mmol VFA/L) were used instead

Batch accumulation experiments were carried out under ammonia limitation in order to maximize PHA storage. In the fermented molasses produced in stage 1 a very low residual ammonia was observed (ammonia concentration was always below 0.02 N-mmol/L). No ammonia was supplemented to simulated feed.

3.2. Analytical off-line techniques

Cell dry weight was determined using the volatile suspended solids (VSS) procedure described in Standard Methods (APHA, 1995).

Total organic carbon (TOC), from clarified samples (dissolved organic carbon - DOC), was analysed in a Shimadzu TOC automatic analyser. Potassium hydrogen phthalate standards (20 - 500 mgC/L) and sodium hydrogen carbonate and sodium carbonate standards (1 - 25 and 20 - 500 mgC/L) were used to produce the TC and IC calibration curves.

VFA (acetate, propionate, butyrate and valerate) concentrations were determined by high performance liquid chromatography (HPLC) using a Merck-Hitachi chromatographer equipped with a RI detector and Aminex HPX-87H pre-column and column (BioRad, USA). Sulphuric acid 0.01 M was used as eluent at a flow rate of 0.6 mL/min and 50°C operating temperature. The organic acids concentrations were calculated using a standard curve of 25 – 1000 mg/L.

Ammonia concentration was determined using an ammonia gas sensing combination electrode (ThermoOrion 9512). A calibration curve was obtained with NH_4Cl standards (0.01 – 10 mmol N/L).

Polyhydroxyalkanoates were determined by gas chromatography (Albuquerque et al. 2010b). Lyophilized biomass was incubated for methanolysis in chloroform and a 20% sulphuric acid in methanol solution. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) of each sample was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID Varian CP-3800). A ZBWax-Plus column (60 m, 0.53 mm ID, 1.0 µm film thickness) was used at a constant pressure of 14.5 psi. Split injection at 280ºC with a split ratio of 10 was used. The oven temperature program was as follows: initial 40ºC; then 20ºC/min until 100ºC; then 3ºC/min until 175ºC and finally 20ºC/min until 220ºC. The detector temperature was set at 230ºC. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined using P(HB-HV) (88% / 12%) (Sigma) standards, corrected using a heptadecane internal standard.

3.3. Experimental data

The batch experiments used throughout this thesis are compiled in Table 3.2. Five of these experiments were fed with the clarified fermented molasses (FM I-V) and two were fed with the simulated feed (SF I-II).

All the experiments were performed following a pulse wise feeding strategy, except for FM I where only one pulse was added. This strategy was chosen in order to prevent inhibition by VFA which occurs above 90 C-mmol VFA/L as was previously reported for this culture (Albuquerque et al., 2007). The end of a pulse was detected by the dissolved oxygen (pO_2) on-line data since O_2 consumption rapidly decreases when substrates are fully depleted causing a sudden rise in pO_2. Figure 3.2 shows the pO_2 behavior over pulses for one of the batch experiments (FM V) as also the respective carbon consumption and PHA production (off-line data). Once pH was not controlled, its' time profile is also represented in Figure 3.2.

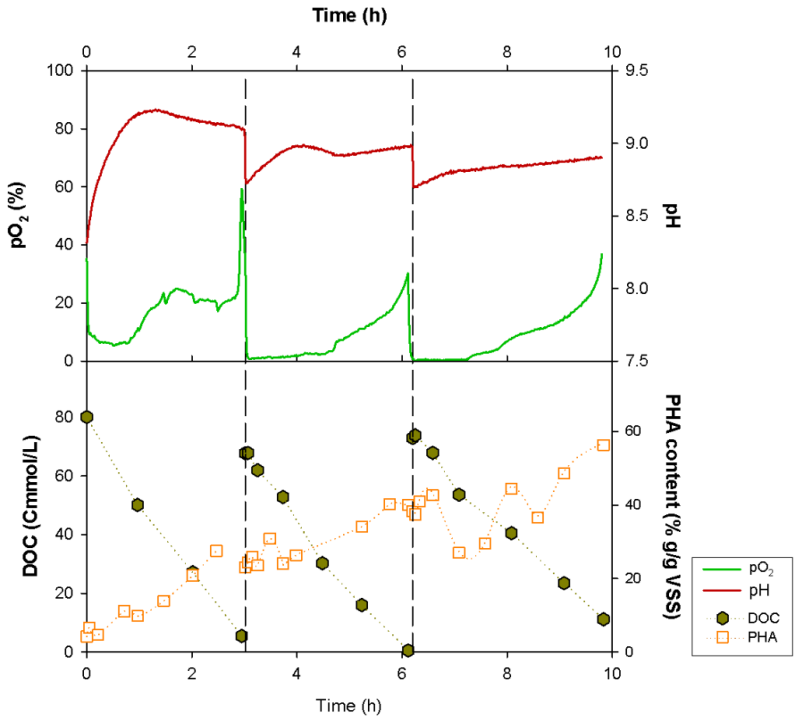


Figure 3.2: Dissolved oxygen (pO_2) as indicator of VFA exhaustion (end of a pulse). On-line (pH and pO_2) and off-line (DOC and PHA) data for the experiment FM V are represented, respectively, on the top and bottom graphs.

In the experiments fed with clarified fermented molasses, it was detected that not only the VFA was consumed but also the consumption of a non identifiable lower fraction of DOC was obtained (Figure 3.3A). This fraction, so-called FA, was then calculated by the difference between the consumed DOC and the total consumed VFA. It was observed that FA fraction, present in much less amount, had the same time profile than VFA over pulses (Figure 3.3B) and, from now on, it will be treated as an independent carbon source.

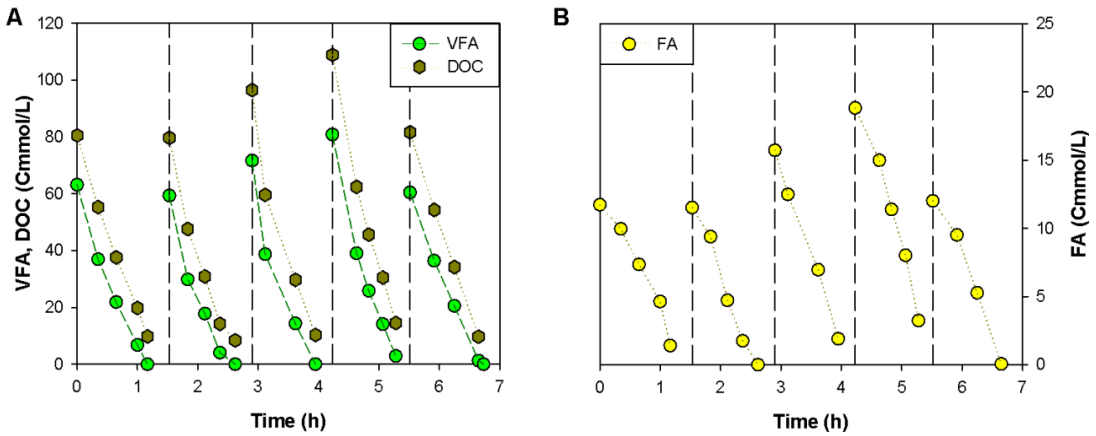


Figure 3.3: Carbon consumption on PHA batch accumulation experiment FM III. (A) VFA are not the only carbon sources consumed over pulses. (B) FA includes the remaining consumed DOC. (dashed lines mark the beginning of a new pulse).

Table 3.2: Summary of PHA batch accumulation experiments performed either with the fermented sugar cane molasses (FM) or the simulated feed (SF).

Reactor	Batch	SBR time of enrichment (days)	PHA batch accumulation experiments						
				Total substrate C-mmol/L	HAc C-mmol/L	HProp C-mmol/L	HBut C-mmol/L	HVal C-mmol/L	FA C-mmol/L
1	FM I	68	pulse 1	100	75	10	15	0	0
1	FMII	247	pulse 1	110	51	13	22	5	19
			pulse 2	123	55	15	25	6	22
			pulse 3	120	56	7	29	6	22
1	FMII	324	pulse 1	73	36	17	7	1	12
			pulse 2	71	36	17	6	0	12
			pulse 3	89	44	21	7	1	16
			pulse 4	101	49	23	9	1	19
			pulse 5	74	37	17	7	1	12
1	FMIV	364	pulse 1	116	47	7	34	5	23
			pulse 2	124	51	7	37	5	24
			pulse 3	128	53	7	39	5	24
			pulse 4	110	35	8	40	6	21
2	SFI	549	pulse 1	94	57	14	19	4	-
			pulse 2	84	50	13	17	4	-
			pulse 3	81	48	13	16	4	-
			pulse 4	78	51	13	11	3	-
			pulse 5	61	37	10	12	2	-
2	SFII	584	pulse 1	83	50	13	17	3	-
			pulse 2	81	49	12	17	3	-
			pulse 3	87	53	13	18	3	-
			pulse 4	85	52	13	16	4	-
2	FM V	860	pulse 1	100	50	9	17	4	20
			pulse 2	101	55	10	18	4	14
			pulse 3	100	50	9	17	3	21

3.3.1. Calculation of kinetic and stoichiometric yields

The consumption rate for each substrate (acetate, propionate, butyrate, valerate and FA) and the production rate for each monomer (HB and HV) were determined by adjusting a linear function to the respective specific concentration (normalized to active biomass). This calculation was performed for each pulse since all experiment were carried out under a pulsewise feeding strategy. For example, Figure 3.4 shows the fitting of linear functions to the experimental data (substrate and product concentrations over time) for each pulse in FMII experiment. Table 3.3 compiles the calculated measured fluxes and the respective correlation coefficient (R^2) for the adjusted linear function for all batch experiments. It should be noted that this method of flux calculation is feasible given the observed linearity in the concentration time profiles within each pulse and the negligible biomass growth rate.

PHA content was calculated as a percentage of VSS on a mass basis (eq. 3.1), where VSS includes active biomass (X) and PHA. Active biomass was calculated by subtracting PHA from VSS.

$$\%\ \mathrm{PHA} = \frac{\mathrm{g\ PHA}}{\mathrm{g\ VSS}} \times 100 \tag{3.1}$$

The composition of PHA (HB:HV) usually is calculated by the HB and HV concentrations determined by GC. However, in this thesis PHA composition were calculated, on a C-mol basis, considering each monomer production rate as a percentage of total PHA production rate (eq. 3.2a and 3.2b). This choice was made to have all calculations performed with the production or substrate uptake rates.

$$\%\ \mathrm{HB} = \frac{r_{HB}}{r_{PHA}} \times 100 \tag{3.2a}$$

$$\%\ \mathrm{HV} = \frac{r_{HV}}{r_{PHA}} \times 100 \tag{3.2b}$$

The observed yields of PHA on substrate were determined by dividing the amount of produced PHA by the total amount of consumed substrate. These yields were calculated either for total VFA (acetate, propionate, butyrate and valerate) or DOC ($Y_{PHA/VFA}$ in C-mol PHA/C-mol VFA or $Y_{PHA/DOC}$ in C-mol PHA/C-mol DOC) because, as stated in the previous section, a part of the remaining DOC was also consumed by the culture. Since the uptake rates of all 5 carbon sources and the production rates for both monomers are linear, the amount of consumed

substrate and the amount of produced PHA were calculated multiplying the respective time by the rate (eq. 3.3). The time of consumption/production had to be taken into account since the 5 carbon sources have different consumption times as can be seen in Figure 3.4.

$$Y_{PHA/DOC} = \frac{r_{HB} \times \Delta t_{HB} + r_{HV} \times \Delta t_{HV}}{\sum_i r_i \times \Delta t_i}; \qquad i = \{\text{Ac,Prop,But,Val,FA}\} \tag{3.3}$$

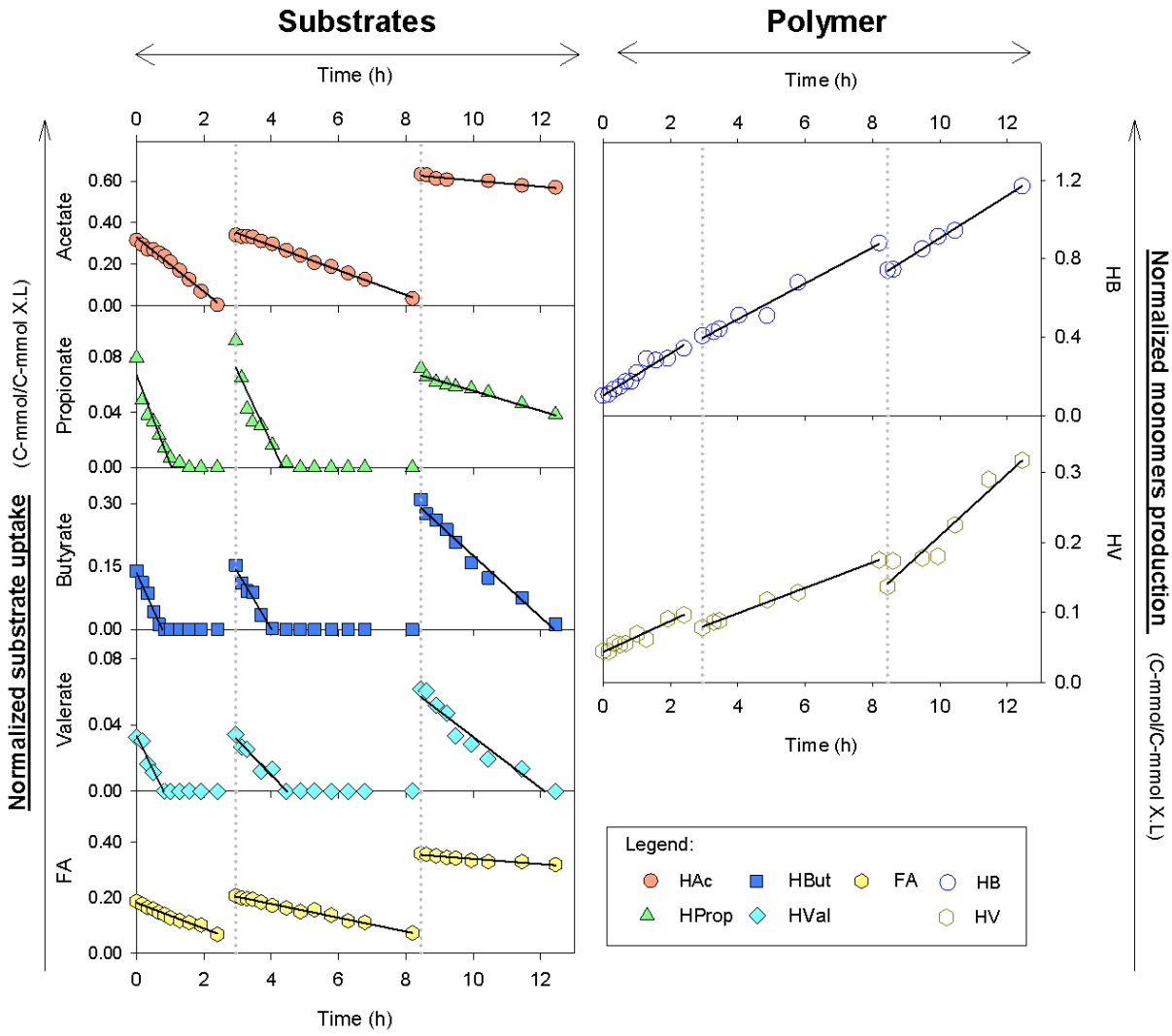


Figure 3.4: Consumption of substrates (acetate, propionate, butyrate, valerate and FA) and production of HB and HV monomers over time for FM II experiment (substrates and monomers concentrations are normalized by active biomass). The symbols represent the normalized concentrations, the lines represent the adjustment of a linear function and dashed lines mark the beginning of a new pulse.

Table 3.3: Kinetic parameters and respective correlation coefficients calculate according to section 3.3 for all batch experiments.

Batch Experiment	Pulse	Measured fluxes											
		rAc		rProp		rBut		rVal		rFA		rHB		rHV	
		(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2	(C-mmol/C-mmol X.h)	R^2
FMI	1	-0.131 ± 0.004	0.99	-0.039 ± 0.004	0.93	-0.108 ± 0.009	0.96	0.000 ± 0.001	-	0.000 ± 0.001	-	0.049 ± 0.002	0.99	0.014 ± 0.001	0.93
FMII	1	-0.130 ± 0.005	0.98	-0.065 ± 0.009	0.92	-0.18 ± 0.01	0.98	-0.042 ± 0.005	0.96	-0.047 ± 0.002	0.98	0.11 ± 0.01	0.93	0.022 ± 0.002	0.93
	2	-0.059 ± 0.001	0.99	-0.05 ± 0.01	0.85	-0.13 ± 0.01	0.97	-0.021 ± 0.003	0.94	-0.025 ± 0.001	0.99	0.091 ± 0.007	0.97	0.018 ± 0.001	0.99
	3	-0.015 ± 0.002	0.92	-0.007 ± 0.001	0.96	-0.073 ± 0.004	0.98	-0.016 ± 0.002	0.94	-0.009 ± 0.001	0.91	0.108 ± 0.007	0.99	0.044 ± 0.008	0.88
FMIII	1	-0.19 ± 0.02	0.98	-0.15 ± 0.01	0.99	-0.11 ± 0.00	1.00	-0.06 ± 0.00	1.00	-0.052 ± 0.006	0.96	0.16 ± 0.03	0.94	0.10 ± 0.01	0.98
	2	-0.21 ± 0.02	0.98	-0.18 ± 0.02	0.98	-0.13 ± 0.00	1.00	0.00 ± 0.00	-	-0.072 ± 0.006	0.98	0.25 ± 0.02	0.99	0.10 ± 0.03	0.89
	3	-0.29 ± 0.03	0.98	-0.20 ± 0.05	0.94	-0.25 ± 0.00	1.00	0.00 ± 0.00	-	-0.096 ± 0.004	0.99	0.35 ± 0.02	0.99	0.12 ± 0.05	0.73
	4	-0.38 ± 0.02	0.99	-0.33 ± 0.03	0.99	-0.13 ± 0.01	0.99	0.00 ± 0.00	-	-0.13 ± 0.01	0.97	0.28 ± 0.05	0.94	0.18 ± 0.04	0.89
	5	-0.29 ± 0.02	0.98	-0.13 ± 0.03	0.91	-0.15 ± 0.00	1.00	0.00 ± 0.00	-	-0.10 ± 0.01	0.98	0.36 ± 0.02	0.99	0.18 ± 0.06	0.81
FMIV	1	-0.16 ± 0.01	0.97	-0.034 ± 0.004	0.94	-0.19 ± 0.01	0.98	-0.034 ± 0.003	0.97	-0.078 ± 0.004	0.98	0.148 ± 0.003	0.99	0.039 ± 0.006	0.94
	2	-0.25 ± 0.01	0.99	-0.037 ± 0.004	0.96	-0.273 ± 0.007	0.99	-0.038 ± 0.003	0.99	-0.105 ± 0.009	0.98	0.32 ± 0.08	0.90	0.09 ± 0.02	0.93
	3	-0.096 ± 0.006	0.99	-0.024 ± 0.001	0.99	-0.14 ± 0.01	0.98	-0.020 ± 0.001	0.99	-0.057 ± 0.007	0.96	0.35 ± 0.06	0.95	0.11 ± 0.02	0.94
	4	-0.115 ± 0.003	0.99	-0.029 ± 0.002	0.98	-0.21 ± 0.02	0.96	-0.030 ± 0.001	0.98	-0.106 ± 0.003	0.99	0.16 ± 0.03	0.91	0.053 ± 0.009	0.93
SFI	1	-0.20 ± 0.01	0.99	-0.066 ± 0.004	0.99	-0.14 ± 0.01	0.98	-0.031 ± 0.003	0.98	-	-	0.21 ± 0.03	0.94	0.08 ± 0.01	0.96
	2	-0.20 ± 0.02	0.95	-0.070 ± 0.007	0.96	-0.13 ± 0.01	0.99	-0.030 ± 0.003	0.98	-	-	0.22 ± 0.01	0.99	0.08 ± 0.01	0.90
	3	-0.18 ± 0.01	0.95	-0.075 ± 0.007	0.96	-0.14 ± 0.01	0.97	-0.031 ± 0.004	0.96	-	-	0.29 ± 0.02	0.97	0.13 ± 0.02	0.95
	4	-0.16 ± 0.02	0.91	-0.053 ± 0.005	0.98	-0.05 ± 0.01	0.94	-0.012 ± 0.002	0.98	-	-	0.28 ± 0.01	0.99	0.09 ± 0.01	0.91
	5	-0.131 ± 0.008	0.98	-0.073 ± 0.008	0.97	-0.08 ± 0.02	0.93	-0.019 ± 0.000	1.00	-	-	0.15 ± 0.02	0.97	0.09 ± 0.02	0.89
SFII	1	-0.182 ± 0.009	0.98	-0.072 ± 0.007	0.94	-0.127 ± 0.004	0.99	-0.032 ± 0.003	0.98	-	-	0.118 ± 0.004	0.99	0.05 ± 0.01	0.85
	2	-0.15 ± 0.01	0.96	-0.065 ± 0.007	0.93	-0.129 ± 0.008	0.98	-0.031 ± 0.003	0.97	-	-	0.17 ± 0.01	0.97	0.048 ± 0.007	0.87
	3	-0.18 ± 0.09	0.99	-0.064 ± 0.007	0.93	-0.15 ± 0.01	0.98	-0.052 ± 0.006	0.97	-	-	0.18 ± 0.02	0.96	0.093 ± 0.008	0.98
	4	-0.07 ± 0.01	0.96	-0.063 ± 0.008	0.96	-0.182 ± 0.009	0.99	-0.04 ± 0.01	0.94	-	-	0.17 ± 0.03	0.97	0.06 ± 0.02	0.92
FMV	1	-0.180 ± 0.015	0.95	-0.054 ± 0.004	0.96	-0.13 ± 0.02	0.88	-0.04 ± 0.01	0.82	-0.049 ± 0.003	0.99	0.13 ± 0.02	0.92	0.021 ± 0.003	0.92
	2	-0.15 ± 0.02	0.89	-0.051 ± 0.004	0.96	-0.13 ± 0.01	0.93	-0.039 ± 0.005	0.93	-0.10 ± 0.02	0.95	0.14 ± 0.02	0.93	0.013 ± 0.003	0.81
	3	-0.130 ± 0.014	0.92	-0.046 ± 0.003	0.98	-0.10 ± 0.01	0.91	-0.026 ± 0.002	0.97	-0.020 ± 0.004	0.84	0.13 ± 0.03	0.88	0.021 ± 0.002	0.97

Table 3.4: PHA related parameters (yields on substrate ($Y_{PHA/S}$), content (PHA %) and composition (HB:HV)) for the 7 batch experiments performed.

Batch	Pulse	$Y_{PHA/S}$		PHA_i	PHA	PHA composition
		C-mol PHA/C-mol VFA	C-mol PHA/C-mol DOC	(%)	(%)	HB:HV
FM I	1	0.37 ± 0.02	0.37 ± 0.02	1	**21**	78:22
FMII	1	0.54 ± 0.05	0.45 ± 0.04	8	32	83:17
	2	0.89 ± 0.07	0.74 ± 0.05	30	57	83:17
	3	1.4 ± 0.1	1.3 ± 0.1	45	**58**	71:29
FMII	1	0.69 ± 0.09	0.61 ± 0.08	4	23	62:38
	2	1.0 ± 0.1	0.8 ± 0.1	18	33	72:28
	3	1.0 ± 0.1	0.8 ± 0.1	23	42	74:26
	4	0.6 ± 0.1	0.54 ± 0.08	34	52	61:39
	5	1.2 ± 0.2	1.0 ± 0.1	48	**62**	67:33
FMIV	1	0.60 ± 0.04	0.48 ± 0.03	2	25	79:21
	2	0.7 ± 0.1	0.6 ± 0.1	24	47	78:22
	3	1.6 ± 0.2	1.4 ± 0.2	20	57	76:24
	4	0.7 ± 0.1	0.51 ± 0.07	58	**75**	75:25
SFI	1	0.9 ± 0.1		14	31	73:27
	2	0.94 ± 0.08		30	41	73:27
	3	1.1 ± 0.1		37	47	69:31
	4	1.3 ± 0.1		47	55	76:24
	5	1.0 ± 0.1		56	**61**	64:36
SFII	1	0.51 ± 0.04		13	32	72:28
	2	0.89 ± 0.07		31	51	78:22
	3	1.0 ± 0.3		45	59	66:34
	4	1.1 ± 0.2		56	**68**	73:27
FM V	1	0.49 ± 0.06	0.42 ± 0.05	4	28	86:14
	2	0.62 ± 0.08	0.48 ± 0.06	23	41	92:8
	3	0.7 ± 0.1	0.6 ± 0.1	39	**57**	86:14

3.4. Conclusions

The relevant information to take for the further chapters of this thesis is:

- MMC PHA production from fermented sugar cane molasses occurs in a 3 stage process;
- The fermented sugar cane molasses is a real and complex media;
- A VFA-rich stream is obtained by an acidogenic fermentation of the sugar cane molasses and next used as substrate for both sludge enrichment (2nd stage) and PHA production (3rd stage) stages;

- The PHA production were made following a pulse wise feeding strategy and ammonia limiting conditions;
- All pulses were treated individually in terms of kinetic and stoichiometric parameters;
- Yields and PHA composition were calculated always using the substrate uptake and production rates.

4 Development of the metabolic network

PHA production processes by MMC from fermented low-cost substrates are highly variable processes in comparison to those using pure cultures or synthetic substrates. The application of metabolic models may contribute to understand the effect of using such complex substrates on these systems. Currently, the available metabolic networks are only able to describe processes with no more than two VFA and for this reason they are not suitable to those using complex substrates.

In this Chapter, a metabolic network for complex mixtures of VFA was adapted from a previous published network (Dias et al., 2008) *developed for the simultaneous uptake of acetate and propionate. The current model includes generic reactions for the uptake of VFA with odd and even number of carbons. The resulting model includes 2 additional parameters to describe the number of carbons of odd and even VFA. The material balances and theoretical yields were derived and compared to those obtained for acetate and propionate uptake from the model published by Dias et al. (2008). The current model is able to describe the PHA production process from the uptake of single to complex mixtures of VFA.*

The metabolic network developed in this chapter will be applied and further validated in the following chapters and will support the development of new computational tools applied to PHA production process from fermented sugar cane molasses.

4.1. Background

Activated sludge model no. 3 (ASM3) was the first mathematical model that considered the occurrence of storage compounds in mixed microbial cultures (MMC) (Gujer et al., 1999). Besides aerobic or anoxic storage of COD, ASM3 also describes sludge production, nitrification and denitrification processes in a heterogeneous group of organisms (heterotrophic and autotrophic) (Gujer et al., 1999). A simplified ASM3 was applied to aerobic heterotrophic storage of acetate in the form of polyhydroxybutyrate (PHB) by eliminating the autotrophic organism's contribution (Krishna and Van Loosdrecht, 1999). Since then, this simplified ASM3 has been used as reference for the development of improved mathematical models of mixed microbial communities.

Aiming to achieve a more realistic model describing the storage of PHB by MMC under dynamic conditions, Beun et al. (2000b) proposed a metabolic model based on seven metabolic reactions. Since the MMC composition was frequently unknown, the metabolic reactions adopted were from an existing metabolic model that describes the stoichiometry of PHB metabolism by a pure culture of *Paracoccus pantotrophus* under dynamic substrate supply (van Aalst-van Leeuwen et al., 1997). Metabolic models have the advantage of determining yield and maintenance coefficient based on the same metabolic parameters. As such, the MMC metabolic model developed by Beun et al. (2000a), and subsequently improvements (Beun et al., 2000b, 2002), allow the calculation of theoretical yields and maintenance coefficients for polyhydroxyalkanoates (PHA) production process. Based on these models, several others were developed in the following years (Dias et al., 2005; Johnson et al., 2009b; van Loosdrecht and Heijnen, 2002; Third et al., 2003). Each of these models had a different purpose but all of them follow the same metabolic reactions highlighting their importance in model development.

Heteropolymeric PHA, i.e., PHA constituted with other monomers (3-hydroxyvalerate – 3HV and 3-hydroxy-2-methylvalerate - 3H2MV) than only 3-hydroxybutyrate (3HB), have better processability comparing with PHB. The HV present in the copolymer P(HB-co-HV) improves biopolymer in terms of mechanical properties like flexibility, tensile strength and toughness (Reis et al., 2003). Motivated by the practical importance of copolymeyrs, Dias and co-workers (2008) extended their previous metabolic model (Dias et al., 2005) to include production of PHA copolymers. They applied this model to two different cultures (enriched under acetate or propionate feeding) and under different feeding conditions (fed with either a single substrate or with mixtures of the two substrates). Good agreement between experimental and predicted results was obtained for those conditions. However, all experiments were performed under

ammonia limitation, i.e., with negligible growth conditions. To cope with this limitation, Jiang et al. (2011) extended the model developed by Dias et al. (2005) in order to describe not only PHA copolymers production but also growth of MMC under propionate feeding, alone or mixed with acetate.

One of the factors that impacts on the process economics is the price of the substrate. The use of renewable carbon sources (based on agriculture or industrial wastes) for the production of PHA contributes to lower process costs. Also, associated with biodegradability of PHA, allows a sustainable closed cycle (Braunegg et al., 1998). In 2000's decade, several researchers evaluated the use of low-costs substrates (e.g. food waste, molasses, wastewater, reviewed by (Serafim et al., 2008a)) for the PHA production using MMC. Usually low-cost substrates are carbohydrate-rich and MMC cannot store PHA directly from them (they produce glycogen instead); thus, a previous anaerobic fermentation is required to convert carbohydrates into volatile fatty acids (VFA) (reviewed by Dias et al., 2006). The fermented low-cost substrates can be stated as a complex mixture where VFA are the major constituents.

In this kind of systems (involving MMC and fermented low-cost substrates) the process variability is much higher than in systems that are more defined (e.g. use of pure cultures and of synthetic substrates). This variability affects PHA production process efficiency as well as PHA characteristics. Therefore, the use of metabolic models to assess the effect of MMC and real complex wastes on PHA production is highly demanded. Due to fermented wastes contain more than two VFA, the previous mentioned metabolic networks/models could not be applied. Thus, the aim of this chapter is the development of a metabolic network able to describe PHA production by MMC from complex mixtures of VFA which can be further applied to processes where real complex wastes are used (e.g. fermented sugar cane molasses - chapters 5, 6 and 7). The metabolic network was essentially an extension of a previous one (Dias et al., 2008). The most challenging point lies on VFA uptake and, considering the different metabolisms, lumped even and odd fatty acids uptake reactions were assumed. Based on the metabolic network proposed, material balances and theoretical yields were defined and, for acetate and propionate mixtures, compared with those previously defined by Dias et al. (2008).

4.2. Metabolic network

4.2.1. Biochemical reactions

The complete list of biochemical reactions adopted in this work is listed in Table 4.1. Figure 4.1 shows a schematic representation of the metabolic network. All reactions are expressed on a carbon-mole basis. VFA molecules (with an odd or even n number of carbons) are taken up by the cells through active transport, requiring one mole of ATP per mole of VFA (Gottschalk, 1986). Once inside the cell, VFA are first activated to acyl-CoA molecules. Acetate and propionate are activated directly to acetyl-CoA and propionyl-CoA. For simplicity we assume that the remaining VFA pass through β-oxidation pathway to be converted to acetyl-CoA and propionyl-CoA. In this pathway, VFA with even number of carbons are all converted to acetyl-CoA (see R_{E_FA}, table 4.1) and those with odd number of carbons are converted to acetyl-CoA molecules and one molecule of propionyl-CoA that remains after the last turn of the β-oxidation cycle (Kunau et al., 1995; Steinbüchel and Lütke-Eversloh, 2003) (see R_{O_FA}, table 4.1). This simplification is not critical for modelling purposes because the net material, energetic and reducing power balances of the β-oxidation of substrates into acetyl-CoA and propionyl-CoA are identical to those associated to the acyl-CoA conversion into PHA monomer (3-hydroxyacyl-CoA) followed by their conversion into acetyl-CoA and propionyl-CoA. Another mole of ATP is associated to the conversion of one mole of intracellular VFA into acetyl-CoA and propionyl-CoA. The lumped uptake and activation reactions of the individual substrates (R_{E_FA} and R_{O_FA}) are listed in Table 4.1 and the particular cases for acetate, propionate, butyrate and valerate are presented in Table 4.2. Further, one needs to consider that a portion of the propionyl-CoA produced may be converted to acetyl-CoA (Lemos et al., 2006). The net reaction of this pathway is represented by the flux R_3.

For PHA synthesis, acetyl-CoA and propionyl-CoA are reduced and condensed to produce a polymer containing different 3-hydroxyalkanoates (3HA) monomers. However, in the process studied in this thesis only 3HB (R_{PHB}) and 3HV (R_{PHV}) were detected. The fraction of each PHA monomer produced by the cells depends on the fluxes of acetyl-CoA and propionyl-CoA available for polymer production. For modelling purpose it is more convenient to express the various PHA fractions in terms of equivalent reduced and condensed monomers, namely acetyl-CoA* (R_9) and propionyl-CoA* (R_{10}). The PHA formation process from these monomers has no further demand for energy or reducing power.

Biomass growth can derive from both acetyl-CoA and propionyl-CoA precursors. The energy required for the production of 1 C-mol of biomass (X) from acetyl-CoA or propionyl-CoA is

represented by parameters K_1 and K_2, which were estimated to be 1.7 mol ATP/C-mol X (Gommers et al. 1988) and 1.38 mol ATP/C-mol X respectively (Zeng et al., 2003). The reaction stoichiometric coefficients of biomass growth from these precursors were determined through stoichiometric and redox balancing by checking firstly oxygen atoms with water and therefore hydrogen atoms with $NADH_2$. The final reactions are shown in R_4 and R_5. The biomass formula was assumed to be $CH_{1.4}O_{0.4}N_{0.2}$, as proposed by (Henze et al., 1995).

Acetyl-CoA is catabolized to CO_2 through the TCA (R_6) (Gottschalk, 1986). The catabolism of propionyl-CoA is assumed to occur via acetyl-CoA, with R_3 followed by R_6. Oxidative phosphorylation is responsible for energy production and the amount of ATP generated per mole of $NADH_2$ oxidized is expressed by the P/O ratio, δ, which represents the efficiency of oxidative phosphorylation (R_8). In addition to the energetic transformations described above, energy is also required for cell maintenance (R_7).

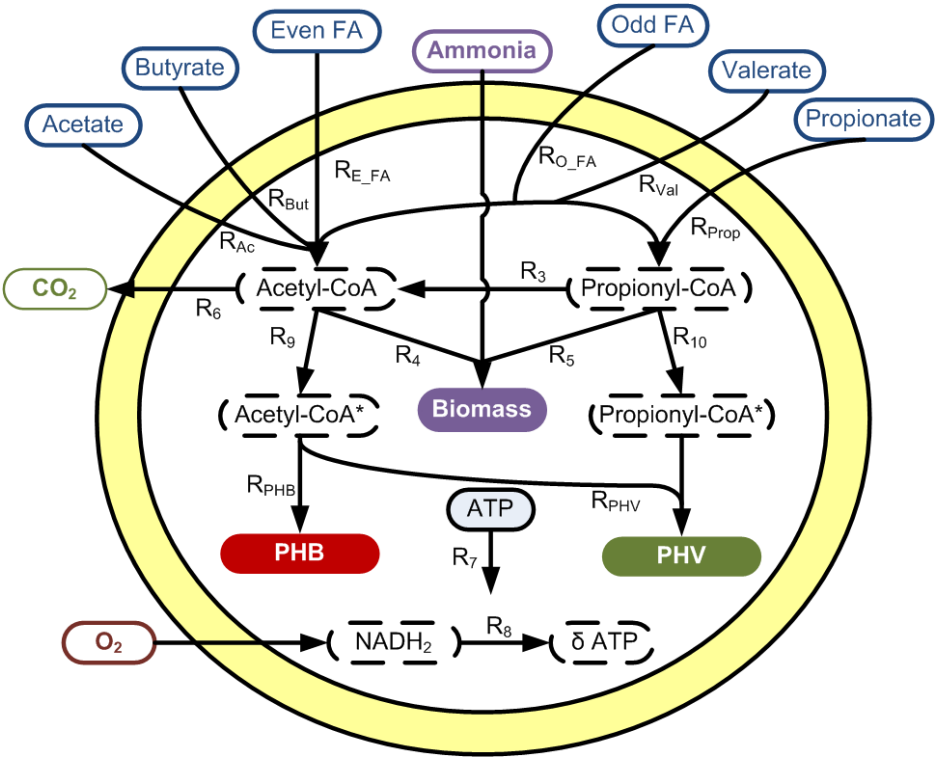


Figure 4.1: Schematic representation of the metabolic model for the PHA production process from fermented sugar cane molasses by mixed microbial cultures under aerobic conditions. Blue stands for the carbon sources present in fermented molasses and dark long dash stands for the intermediate metabolites.

Table 4.1: Metabolic reactions adopted for PHA production process by mixed microbial cultures using mixtures of volatile fatty acids.

Reactions	Description	Stoichiometry
R_{E_FA}	Even FA uptake	$CH_2O_{2/n} + \frac{2}{n} \cdot ATP + \frac{n-4}{2 \cdot n} \cdot H_2O \rightarrow CHO_{1/2} + \frac{n-2}{n} \cdot NADH_2$
R_{O_FA}	Odd FA uptake	$CH_2O_{2/n} + \frac{2}{n} \cdot ATP + \frac{n-5}{2 \cdot n} \cdot H_2O \rightarrow \frac{n-3}{n} \cdot CHO_{1/2} + \frac{3}{n} \cdot CH_{4/3}O_{1/3} + \frac{n-3}{n} \cdot NADH_2$
R_3	Propionyl-CoA convertion to acetyl-CoA	$\frac{3}{2} \cdot CH_{4/3}O_{1/3} + H_2O \rightarrow CHO_{1/2} + \frac{3}{2} \cdot NADH_2 + \frac{1}{2} \cdot CO_2$
R_4	Growth on acetyl-CoA	$1.27 \cdot CHO_{1/2} + 0.2 \cdot NH_3 + K_1 \cdot ATP + 0.3 \cdot H_2O \rightarrow CH_{1.4}N_{0.2}O_{0.4} + 0.53 \cdot NADH_2 + 0.27 \cdot CO_2$
R_5	Growth on propionyl-CoA	$1.06 \cdot CH_{4/3}O_{1/3} + 0.2 \cdot NH_3 + K_2 \cdot ATP + 0.17 \cdot H_2O \rightarrow CH_{1.4}N_{0.2}O_{0.4} + 0.47 \cdot NADH_2 + 0.06 \cdot CO_2$
R_6	Catabolism (TCA cycle)	$CHO_{1/2} + \frac{3}{2} \cdot H_2O \rightarrow CO_2 + 2 \cdot NADH_2 + \frac{1}{2} \cdot ATP$
R_7	Maintenance	$ATP \rightarrow m_{ATP}$
R_8	Oxidative phosphorylation	$NADH_2 + \frac{1}{2} \cdot O_2 \rightarrow H_2O + \delta \cdot ATP$
R_9	Production of HB precursor (acetyl-CoA*)	$CHO_{1/2} + \frac{1}{4} \cdot NADH_2 \rightarrow CH_{3/2}O_{1/2}$
R_{10}	Production of HV precursor (acetyl-CoA*)	$CH_{4/3}O_{1/3} + \frac{1}{6} \cdot NADH_2 \rightarrow CH_{5/3}O_{1/3}$
R_{PHB}	PHB production	$CH_{3/2}O_{1/2} \rightarrow PHB$
R_{PHV}	PHV production	$\frac{2}{5} \cdot CH_{3/2}O_{1/2} + \frac{3}{5} \cdot CH_{5/3}O_{1/3} \rightarrow PHV$

Table 4.2: Reactions for particular VFA: acetate, propionate, butyrate and valerate (predominant VFA presents in fermented sugar cane molasses). These reactions are obtained replacing the n in R_{E_FA} and R_{O_FA} (Table 4.1) by the respective number of carbon of each VFA.

Reactions	Description	Stoichiometry
R_{Ac}	Acetate uptake (n=2)	$CH_2O + ATP \rightarrow CHO_{1/2} + \frac{1}{2}\cdot H_2O$
R_{Prop}	Propionate uptake (n=3)	$CH_2O_{2/3} + \frac{2}{3}\cdot ATP \rightarrow CH_{4/3}O_{1/3} + \frac{1}{3}\cdot H_2O$
R_{But}	Butyrate uptake (n=4)	$CH_2O_{1/2} + \frac{1}{2}\cdot ATP \rightarrow CHO_{1/2} + \frac{1}{2}\cdot NADH_2$
R_{Val}	Valerate uptake (n=5)	$CH_2O_{2/5} + \frac{2}{5}\cdot ATP \rightarrow \frac{2}{5}\cdot CHO_{1/2} + \frac{3}{5}\cdot CH_{4/3}O_{1/3} + \frac{2}{5}\cdot NADH_2$

4.2.2. Steady state material and energetic balancing of intracellular intermediates

Overall, the metabolic network has 10+k reactions (k representing the number of VFA evolved in the substrates uptake processes), 6 intracellular metabolites (acetyl-CoA, propionyl-CoA, acetyl-CoA*, propionyl-CoA*, ATP, $NADH_2$), 2+k input substrates (O_2, ammonia, even and odd fatty-acids) and 4 end-products (biomass, PHB, polyhydroxyvalerate (PHV) and CO_2). Steady-state material balances for the 6 intracellular metabolites (dashed in Figure 4.1) results in the following system of 6 algebraic equations:

$$r_{Ac} + r_{But} + r_{E\_FA} + \frac{2}{5}\cdot r_{Val} + \frac{(n_{FA}-3)}{n_{FA}}\cdot r_{O\_FA} + r_3 - 1.27\cdot r_4 - r_6 - r_9 = 0 \quad (4.1)$$

$$r_{Prop} + \frac{3}{5}\cdot r_{Val} + \frac{3}{n_{FA}}\cdot r_{O\_FA} - \frac{3}{2}\cdot r_3 - 1.06\cdot r_5 - r_{10} = 0 \quad (4.2)$$

$$r_9 - r_{PHB} - \frac{2}{5}\cdot r_{PHV} = 0 \quad (4.3)$$

$$r_{10} - \frac{3}{5}\cdot r_{PHV} = 0 \quad (4.4)$$

$$-r_{Ac} - \frac{2}{3}\cdot r_{Prop} - \frac{1}{2}\cdot r_{But} - \frac{2}{5}\cdot r_{Val} - \frac{2}{n_{FA}}\cdot r_{FA} - K_1\cdot r_4 - K_2\cdot r_5 + \frac{1}{2}\cdot r_6 - r_7 + \delta\cdot r_8 = 0 \quad (4.5)$$

$$\frac{1}{2}\cdot r_{But} + \frac{2}{5}\cdot r_{Val} + \frac{(n_{FA}-2)}{n_{FA}}\cdot r_{E\_FA} + \frac{(n_{FA}-3)}{n_{FA}}\cdot r_{O\_FA} + \frac{3}{2}\cdot r_3 + 0.53\cdot r_4 + 0.47\cdot r_5 + 2\cdot r_6 - r_8 - \frac{1}{4}\cdot r_9 - \frac{1}{6}\cdot r_{10} = 0 \quad (4.6)$$

with n_{FA} representing the average number of carbons of unknown substrates.

4.2.3. Stoichiometric parameters

Likewise to the other metabolic networks, the theoretical yields and maintenance coefficient could be determined as function of metabolic parameters. To determine those were used the material balance equations (eq. 4.1-4.6) previously described. Also, due to the complexity of the substrate, it was necessary to constraint some metabolic parameters and it was adopted a similar approach than the one reported by Dias et al. (2008). Since even and odd carbon sources follow different metabolic pathways, it was defined the ratio, y, of all odd carbon sources flux (r_{Odd}) to total carbon flux (r_S). The equations 4.7 define the calculations needed to define the ratio y.

$$r_{Odd} = r_{Prop} + r_{Val} \tag{4.7a}$$

$$r_{Even} = r_{Ac} + r_{But} + r_{E\_FA} \tag{4.7b}$$

$$r_S = r_{Even} + r_{Odd} \tag{4.7c}$$

$$y = \frac{r_{Odd}}{r_S} \tag{4.7d}$$

Additionally, the number of carbons of even (n_{Even}) and odd (n_{Odd}) substrates were also defined by the weighted average of the number of carbons of each VFA (n_i) by the respective flux (r_i) (see eqs. 4.8a and 4.8b).

$$n_{Even} = \frac{n_{Ac} \cdot r_{Ac} + n_{But} \cdot r_{But} + n_{FA} \cdot r_{E\_FA}}{r_{Even} + r_{Odd}} \tag{4.8a}$$

$$n_{Odd} = \frac{n_{Prop} \cdot r_{Prop} + n_{Val} \cdot r_{Val}}{r_{Even} + r_{Odd}} \tag{4.8b}$$

Finally, it was also used the overall degree of reduction balance (eq. 4.9), where the metabolic parameters defined above were incorporated.

$$\left(6 + \left(\frac{-4}{n_{even}}\right)\right) \cdot (1-y) \cdot r_S + \left(6 + \left(\frac{-4}{n_{odd}}\right)\right) \cdot y \cdot r_S - 2 \cdot r_8 - 4 \cdot r_4 - 4 \cdot r_5 - 4.5 \cdot r_{PHB} - 4.8 \cdot r_{PHV} = 0 \tag{4.9}$$

The resulting expressions for the calculation of the theoretical yields and maintenance coefficient are compiled in Table 4.3.

Reflecting the complexity of the system, the expressions achieved were also complex, which difficult a direct comparison with previously published expressions (Dias et al., 2008). Since the metabolic networks published so far only describe simpler mixtures, acetate plus propionate, just for those cases it was possible to perform this comparison. To accomplish this purpose, values for the metabolic parameters have to be assumed: for K_1, K_2 and δ it was assumed the value 1 to simplify the calculations and for m_{ATP} was used the value 0.02, already published (Beun et al., 2002). In order to comprise a range of values, random values between 0 and 1 were assigned to y. The more complex expressions (Table 4.3), for the simpler VFA mixtures, are coincident with the expressions reported by Dias et al. (2008) (Figure 4.2).

Table 4.3: Theoretical yields on substrate and maintenance coefficient as function of K_1, K_2, δ, y, even and odd carbon numbers.

Process description	Substrate (DOC consumed)
Cell growth on substrates with even carbon number	$Y_{X,Even/S} = \frac{n_{even} \cdot n_{odd} \cdot (1+6\cdot\delta) + 4 \cdot n_{odd}(y+\delta\cdot y-1-\delta) - n_{even} \cdot y \cdot (5+4\cdot\delta)}{n_{even} \cdot n_{odd} \cdot (1.27+4\cdot\delta+2\cdot K_1)}$
Cell growth on substrates with odd carbon number	$Y_{X,Odd/S} = \frac{n_{even} \cdot n_{odd} \cdot (1+6\cdot\delta) + 4 \cdot n_{odd}(y+\delta\cdot y-1-\delta) - n_{even} \cdot y \cdot (5+4\cdot\delta)}{n_{even} \cdot n_{odd} \cdot (0.707+4\cdot\delta+2\cdot K_2)}$
PHB storage	$Y_{PHB/S} = \frac{n_{even} \cdot n_{odd} \cdot (1+6\cdot\delta) + 4 \cdot n_{odd}(y+\delta\cdot y-1-\delta) - n_{even} \cdot y \cdot (5+4\cdot\delta)}{n_{even} \cdot n_{odd} \cdot (1+4.5\cdot\delta)}$
PHV storage	$Y_{PHV/S} = \frac{n_{even} \cdot n_{odd} \cdot (1+6\cdot\delta) + 4 \cdot n_{odd}(y+\delta\cdot y-1-\delta) - n_{even} \cdot y \cdot (5+4\cdot\delta)}{n_{even} \cdot n_{odd} \cdot (0.8+4.8\cdot\delta)}$
Maintenance	$m_s = \frac{2 \cdot n_{even} \cdot n_{odd}}{n_{even} \cdot n_{odd} \cdot (1+6\cdot\delta) + 4 \cdot n_{odd}(y+\delta\cdot y-1-\delta) - n_{even} \cdot y \cdot (5+4\cdot\delta)} \cdot m_{ATP}$

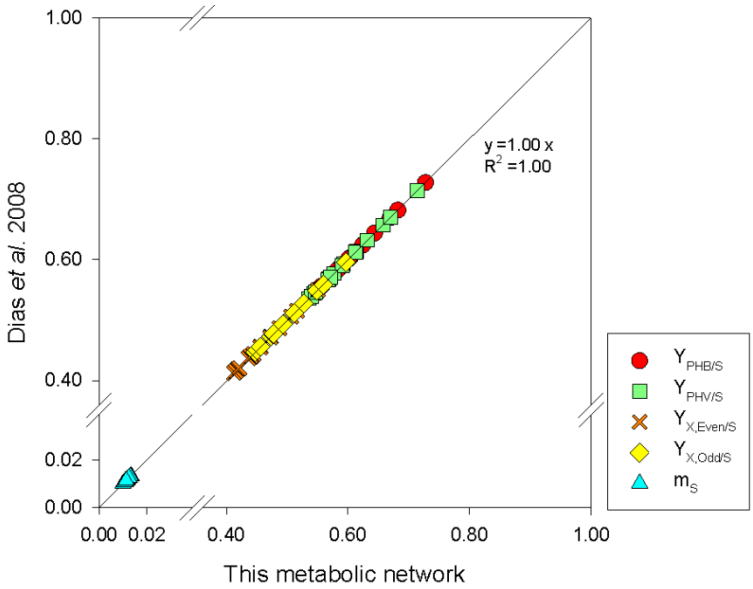


Figure 4.2: Comparison between the theoretical yields and maintenance coefficient calculated by the expressions derived here (Table 4.3) and the expressions derived by Dias et al. (2008). K_1, K_2 and δ values were assumed to be 1 to simplify calculations and m_{ATP} was 0.02. Random values for y were assumed.

4.4. Conclusions

In this chapter, a metabolic network for the uptake of complex mixtures of VFA and production of PHA was developed. This network is an extension of a previous model developed by (Dias et al., 2008) and includes generic reactions for the simultaneous VFA uptake with odd and even number of carbons. The model enables to calculate the theoretical yields for cell growth and PHA production as well as the maintenance coefficients when the composition of the substrate is known whenever the substrate is simpler or more complex.

The current metabolic network is of key importance to validate the computational systems biology tools and models developed in the following chapters.

5 Flux balance analysis

Fermented agro-industrial wastes are potential low cost substrates for polyhydroxyalkanoates (PHA) production by mixed microbial cultures (MMC). The use of complex substrates has however profound implications in the PHA metabolism. In this paper we investigate PHA accumulation using a lumped metabolic model that describes PHA storage from arbitrary mixtures of volatile fatty acids (VFA). Experiments were conducted using synthetic and complex VFA mixtures obtained from the fermentation of sugar cane molasses. Metabolic flux analysis (MFA) and flux balance analysis (FBA) were performed at different stages of culture enrichment in order to investigate the effect of VFA composition and time of enrichment in PHA storage efficiency. Substrate uptake and PHA storage fluxes increased over enrichment time by 70% and 73%, respectively. MFA calculations show that higher PHA storage fluxes are associated to an increase in the uptake of VFA with even number of carbon atoms and a more effective synthesis of hydroxyvalerate (HV) precursors from VFA with odd number of carbons. Furthermore, FBA shows that the key metabolic objective of a MMC subjected to the feast and famine regimen is the minimization of the tricarboxylic acid cycle fluxes. The PHA flux and biopolymer composition (hydroxybutyrate (HB): HV) could be accurately predicted in several independent experiments.

The contents of this chapter were adapted from the publication: Pardelha F, Albuquerque MGE, Reis MAM, Dias JML, Oliveira R. 2012. Flux balance analysis of mixed microbial cultures: application to the production of polyhydroxyalkanoates from complex mixtures of volatile fatty acids. Journal of Biotechnology. DOI: 10.1016/j.biotec.2012.08.017 [1]

5.1. Introduction

Polyhydroxyalkanoates (PHA) are intracellular biopolymers used as carbon and energy reserves by a wide variety of bacteria. These biopolymers have similar properties to petroleum-based plastics with the advantage of being biodegradable, biocompatible and renewable. Nowadays, PHA are produced industrially through cultivation of natural or genetically engineered bacteria. However, current PHA production costs cannot compete with conventional petroleum-based plastics. The use of carbon rich effluents and open mixed microbial cultures (MMC) are being investigated to decrease PHA production costs (Serafim et al., 2008a). Fermented paper mill effluents (Bengtsson et al., 2008), olive oil mill effluents (Beccari et al., 2009), wastewater and surplus sludge from wastewater treatment plants (Mengmeng et al., 2009) are examples of complex substrates that have been recently investigated for low cost PHA production by MMC.

The anaerobic digestion of carbon rich effluents under acidic pH produces a variety of complex volatile fatty acids (VFA) mixtures that can be used as substrate for PHA production by MMC. Sugar cane molasses are a high sugar content by-product (up to 50% dry weight) of the sugar refinery industry that can be converted by anaerobic fermentation to acetate, butyrate, propionate and valerate mixtures with yields up to 0.77 C-mmol VFA/C-mmol sugars (Albuquerque et al., 2007). However, the use of such complex substrates has deep implications in the subsequent PHA production process. The relative composition of VFA mixtures was shown to greatly affect the efficiency of MMC enrichment in PHA-storing organisms, PHA monomeric composition and molecular weight (MW), overall PHA yield and productivity (Albuquerque et al., 2007). Understanding the metabolism of multiple VFA conversion to PHA is thus of paramount importance for process design and optimization towards a more rational use of fermented feedstock for PHA production.

Intracellular regulation of PHA synthesis from mixtures of VFA is considerably more complex than the single substrate scenario. Most studies have been carried out for pure cultures of *Ralstonia eutropha* grown on single carbon sources (Shi et al., 1997; Yu and Si, 2004). Even so, the knowledge gathered for single substrates can be useful to formulate a multiple substrate model. The regulation of PHA synthesis can take place at either the transcription or the enzymatic level or a combination of both depending of the microorganism (Kessler and Witholt, 2001). A study about transcription regulation on PHA formation in *R. eutropha* demonstrated that most of up-regulated proteins during the induction of PHA production are those related to fatty acids biosynthesis for acetate, β-oxidation for propionate and both for levulinic acid (Lee et al., 2009). Moreover, Henderson and Jones (Henderson and Jones, 1997) suggested that a key

enzymatic regulation occurs in TCA cycle, more specifically on citrate synthase activity (Kessler and Witholt, 2001). This enzyme catalyses the condensation of acetyl-CoA to form citrate and coenzyme-A, and is significantly inhibited by $NADH_2$ accumulation. Coenzyme-A produced in this reaction can also reduce the activity of 3-ketothiolase. Thus, the flux of PHA synthesis can be enhanced by blocking the TCA cycle, causing the accumulated acetyl-CoA to be channelled through PHA pathways. This hypothesis was supported by experiments performed with an isocitrate dehydrogenase-leaky mutant of *R. eutropha* which exhibited lower TCA cycle activity and higher PHA synthesis fluxes in comparison with the wild organism (Park and Lee, 1996). Moreover, the addition of formate (a potential source of $NADH_2$) along with glucose was shown to significantly stimulate the production of PHB and decreased the flux of carbon going through the TCA cycle (Henderson and Jones, 1997). The PHB pathway leaked the pool of acetyl-CoA formed by blocking the TCA cycle and consumed the $NADH_2$ generated from formate uptake. Experiments performed with a recombinant strain of *R. eutropha* revealed that the PHB biosynthesis rate is also controlled by 3-ketothiolase while the PHB content is controlled by PHB synthase (Jung and Lee, 2000).

Similar studies for MMC are difficult to perform due to systems' complexity. Metabolic models were developed to better understand PHA accumulation by MMC and to derive new strategies to optimise PHA production (Beun et al., 2000b; Dias et al., 2008; Filipe et al., 2001; Gujer et al., 1999; Jiang et al., 2011a; Johnson et al., 2009b). In this study we formulate a metabolic model for multiple VFA uptake and conversion to PHA, which is a follow up of previously published models (Dias et al., 2005, 2008; Jiang et al., 2011a; Johnson et al., 2009b). The proposed model is the first attempt to describe the simultaneous uptake of more than two carbon sources for PHA production. The model was validated with data of PHA production from fermented sugar cane molasses. Using this model we have performed metabolic flux analysis (MFA) and flux balance analysis (FBA) in order to understand the impact of sludge enrichment strategy and substrate complexity on the PHA metabolism. We also used the metabolic model to predict PHA production from substrate uptake rates.

5.2. Metabolic model

5.2.1. Metabolic Network

The metabolic network used in this Chapter is described in Chapter 4. R_4 and R_5 reactions were not take in account since the growth is negligible in the PHA production experiments.

5.2.2. Steady-state material and energetic balances

Overall, the metabolic network has 8+k reactions (k representing the number of VFA evolved in the substrates uptake processes), 6 intracellular metabolites (acetyl-CoA, propionyl-CoA, acetyl-CoA*, propionyl-CoA*, ATP, $NADH_2$), 1+k input substrates (O_2, even and odd fatty-acids) and 3 end-products (PHB, polyhydroxyvalerate (PHV) and CO_2). Steady-state material balances for the 6 intracellular metabolites results in the following system of 6 algebraic equations:

$$r_{Ac} + r_{But} + r_{E\_FA} + \frac{2}{5}\cdot r_{Val} + \frac{(n_{FA}-3)}{n_{FA}}\cdot r_{O\_FA} + r_3 - r_6 - r_9 = 0 \quad (5.1)$$

$$r_{Prop} + \frac{3}{5}\cdot r_{Val} + \frac{3}{n_{FA}}\cdot r_{O\_FA} - \frac{3}{2}\cdot r_3 - r_{10} = 0 \quad (5.2)$$

$$r_9 - r_{PHB} - \frac{2}{5}\cdot r_{PHV} = 0 \quad (5.3)$$

$$r_{10} - \frac{3}{5}\cdot r_{PHV} = 0 \quad (5.4)$$

$$-r_{Ac} - \frac{2}{3}\cdot r_{Prop} - \frac{1}{2}\cdot r_{But} - \frac{2}{5}\cdot r_{Val} - \frac{2}{n_{FA}}\cdot r_{FA} + \frac{1}{2}\cdot r_6 - r_7 + \delta\cdot r_8 = 0 \quad (5.5)$$

$$\frac{1}{2}\cdot r_{But} + \frac{2}{5}\cdot r_{Val} + \frac{(n_{FA}-2)}{n_{FA}}\cdot r_{E\_FA} + \frac{(n_{FA}-3)}{n_{FA}}\cdot r_{O\_FA} + \frac{3}{2}\cdot r_3 + 2\cdot r_6 - r_8 - \frac{1}{4}\cdot r_9 - \frac{1}{6}\cdot r_{10} = 0 \quad (5.6)$$

with n_{FA} representing the average number of carbons of unknown substrates.

All metabolic reactions but R_7 and R_8 are irreversible. These two reactions comprise the β-oxidation pathway of longer chain VFA into acetyl-CoA and propionyl-CoA. In case of PHA precursors synthesis overflow, the excess is converted back to acetyl-CoA and propionyl-CoA via β-oxidation. Thus,

$$r_i \geq 0, \forall_{i\neq 9,10} \quad (5.7)$$

5.2.3. Metabolic flux calculations

The fermented molasses complex substrate comprises 4 well-defined VFAs (acetate, propionate, butyrate and valerate) and a 5th fatty acid (FA) that lumps all other substrates with unknown composition. Therefore, the metabolic model has 14 metabolic reactions and 6 connectivity equality constraints (Eqs. 5.1-5.6), thus at least 14-6=8 fluxes must be measured in order for the system to be determined. In this work we measure 8 fluxes, fixed 1 (r_7 = m_{ATP} = 0.02 mol ATP/(C-mol.h) (Beun et al., 2000c)) and calculate 5 fluxes. The system becomes then

determined and redundant with 1 degree of freedom. The partition of measured ($\mathbf{v_b}$) and estimated ($\mathbf{v_n}$) fluxes is as follows:

$$\mathbf{v_b} = \left[r_{Ac}, r_{But}, r_{Prop}, r_{Val}, r_{FA}, r_{HB}, r_{HV}, r_5 \right] \tag{5.8}$$

$$\mathbf{v_n} = \left[r_3, r_6, r_8, r_9, r_{10} \right] \tag{5.9}$$

The 5 unknown fluxes were calculated by standard methods of metabolic flux analysis (Stephanopoulos et al., 1998). The central MFA equation is represented by:

$$\mathbf{A} \cdot \mathbf{v} = \mathbf{A_n} \cdot \mathbf{v_n} + \mathbf{A_b} \cdot \mathbf{v_b} = \mathbf{0} \tag{5.10}$$

with $\mathbf{A}$ a 6x14 stoichiometric matrix obtained from Eqs (5.1)-(5.6), $\mathbf{v}$ the vector of fluxes and subscript indexes *b* and *n* denoting the partitions of known and unknown fluxes, respectively. Since the system is determined and redundant, matrix $\mathbf{A_n}$ is non-square and the rank of $\mathbf{A_n}$ correspond to the number of unknown fluxes. In such cases, the unknown fluxes are calculated using the following formula:

$$\mathbf{v_n} = -\mathbf{A_n}^{\#} \cdot \mathbf{A_b} \cdot \mathbf{v_b} \tag{5.11}$$

where $\mathbf{A_n^{\#}}$ is the Moore-Penrose pseudo-inverse matrix of $\mathbf{A_n}$.

The Chi-squared (χ^2) statistical test was implemented to validate the consistency between assumed metabolic constraints (Eqs. 5.1-5.6) and measured flux vectors corrupted by experimental error. More specifically, metabolic flux distribution (MFD) estimations are said to be consistent when the consistency index (h) is below a given threshold χ^2 value (Wang and Stephanopoulos, 1983). The consistency index was calculated by the weighted sum of flux errors according to equation 5.12.

$$h = \mathbf{e}' \cdot \mathbf{Y}^{-1} \cdot \mathbf{e} \tag{5.12}$$

where $\mathbf{e} = \mathbf{A} \cdot \mathbf{v} \neq \mathbf{0}$ due to the experimental error in $\mathbf{v_b}$. The matrix $\mathbf{Y}$ is the variance-covariance matrix of the steady state errors, $\mathbf{e}$, calculated by the propagation of variance-covariance matrix associated to the measured fluxes, $\mathbf{v_b}$. For more details about the consistency index method and the calculation of $\mathbf{Y}$ see Stephanopoulos *et al.* (Stephanopoulos et al., 1998).

5.2.4. Flux Balance Analysis

Flux balance analysis (FBA) is a technique that applies linear programming to calculate MFDs that maximise or minimise a predefined objective function under the constraint of steady state material balances (Eq. 5.10) (Chaganti et al., 2011). As opposed to MFA, FBA can be applied to undetermined systems. However, more realistic MFDs can be calculated if some measured

fluxes are imposed as constraints to FBA optimisations. We have implemented a FBA code in MATLAB (using the *fmincon* function) that solves the following optimization problem.

$$\max_{\mathbf{v}} \quad Z = \mathbf{c} \cdot \mathbf{v}$$
$$\text{s.t.} \quad \begin{cases} \mathbf{0} = \mathbf{A_n} \cdot \mathbf{v_n} + \mathbf{A_b} \cdot \mathbf{v_b} \\ \mathbf{v_j} \geq \mathbf{0} \\ \mathbf{b} - \boldsymbol{\sigma}_\mathbf{b} \leq \mathbf{v_b} \leq \mathbf{b} + \boldsymbol{\sigma}_\mathbf{b} \end{cases} \tag{5.13}$$

The performance index Z is the objective function and the degrees of freedom are the metabolic fluxes, $\mathbf{v}$. The elements of vector $\mathbf{c}$ take the values -1 or +1 to minimize or maximize a given target metabolic flux or 0 otherwise. This optimisation problem is subject to the steady state material balance constraints ($\mathbf{0} = \mathbf{A_n} \cdot \mathbf{v_n} + \mathbf{A_b} \cdot \mathbf{v_b}$), to the flux irreversibility constraints ($\mathbf{v_j \geq 0}$) and to flux measurement constraints ($\mathbf{b} - \boldsymbol{\sigma}_\mathbf{b} \leq \mathbf{v_b} \leq \mathbf{b} + \boldsymbol{\sigma}_\mathbf{b}$) with $\mathbf{b}$ a measurement of $\mathbf{v_b}$ and $\boldsymbol{\sigma_b}$ a vector of measurement error standard deviations. In the present study we have set as measurement constraints the measured substrates fluxes.

$$\mathbf{v_b} = \left[r_{Ac}, r_{But}, r_{Prop}, r_{Val}, r_{FA} \right] \tag{5.14}$$

FBA then calculates all other fluxes including the HB and HV fluxes:

$$\mathbf{v_n} = \left[r_3, r_6, r_7, r_8, r_9, r_{10}, r_{HB}, r_{HV} \right] \tag{5.15}$$

To validate FBA calculations the estimated HB and HV fluxes are compared with the corresponding experimental values.

5.3. Material and methods

The description of all material and methods relevant for this work is presented on chapter 3 of this Ph.D. thesis. The batch experiments used in the present chapter were FMII, FM III and FMIV for fermented molasses and SFI and SFII for synthetic feed.

5.4. Results and discussion

5.4.1. PHA accumulation experiments

Three batch experiments (FMII, FMIII and FMIV) fed with fermented molasses and two batch experiments (SFI and SFII) fed with synthetic VFA mixture were carried out with MMC enriched in PHA accumulators (selected in the SBR reactor as described in the materials and methods section). In all experiments, co-polymers of hydroxybutyrate (HB) and hydroxyvalerate (HV), P(HB-co-HV), were produced (confirmed by GPC-SEC, results not shown). The maximum PHA

content achieved was relatively high, between 58% and 75%, with the highest PHA content obtained in the experiment FMIV using fermented molasses (see Table 3.4). Similar PHA contents stored by MMC fed with fermented molasses were reported by Albuquerque et al. (2010b) . Higher PHA contents were reported by, Johnson et al. (2009) and Jiang et al. (2011b) (89% (w/w) and 90% (w/w) respectively) using, however, single carbon sources (acetate and lactate, respectively) in both enrichment and accumulation stages.

Beside VFA, PHA is also produced from a residual unknown carbon source present in the fermented molasses. Indeed, the experimental PHA/VFA yields considering only measured VFA (acetate, propionate, butyrate and valerate) are in the range 0.73-0.81 C-mol PHA/C-mol VFA. When calculated on the basis of measured dissolved TOC (DOC), they decrease to 0.58-0.66 C-mol PHA/C-mol DOC, which is a clear indication that PHA is also produced from unidentified fatty acids (FA). Given the relatively high impact in the yields, this unknown fraction of carbon was also considered in the metabolic flux analysis shown below.

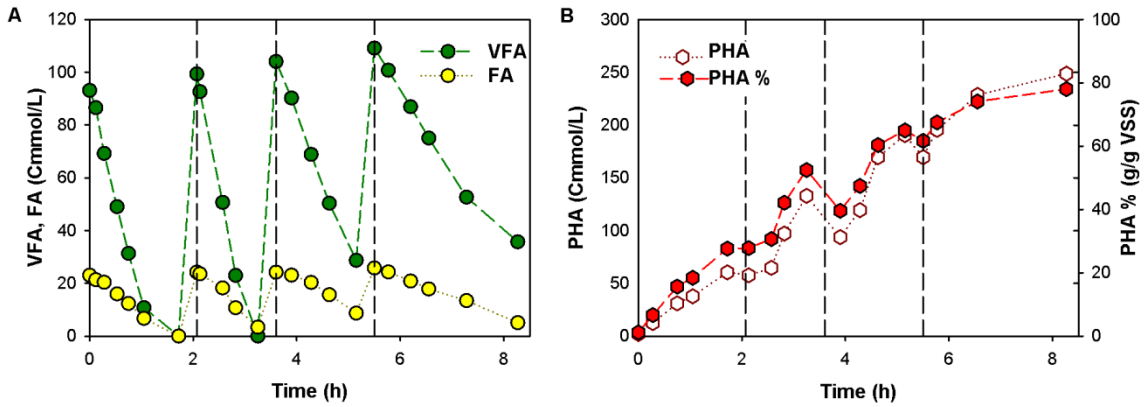


Figure 5.1: PHA batch accumulation experiment FMIV. A) Carbon consumption over pulses. VFA includes sum of acetate, propionate, butyrate and valerate; FA includes the remaining DOC. B) PHA production over pulses (dashed line marks the beginning of a new pulse).

Figure 5.1 shows the measured time profile of VFA (comprising acetate, propionate, butyrate and valerate), the unknown FA (DOC excluding VFA), PHA fraction (f_{PHA}) and PHA content for the batch experiment with highest PHA content (FMIV). In this, as in all other experiments, the substrate was fed pulse-wise. The experimental data was segmented in pulses as illustrated in Figure 5.1. For each pulse, experimental fluxes of acetate, propionate, butyrate, valerate, FA, HB and HV were determined from the corresponding concentrations time profile as described in chapter 3. The overall results are presented in Table 3.3. It should be noted that the measured concentrations time profiles show similar directions of variations in all experiments but the

magnitude of reaction rates changed significantly from pulse-to-pulse and from batch-to-batch as shown in Table 3.3.

5.4.2. Metabolic flux analysis

5.4.2.1. Pulse-to-pulse metabolic fluxes variability

The intracellular fluxes were calculated by metabolic flux analysis for each pulse of the different experiments using the previously described MFA method. Figure 5.2 A illustrates those results for the 2nd pulse of FMIV experiment. Table 5.1 shows the consistency index obtained for each pulse and the inverse of the chi-square, *χ2*, cumulative distribution for 1 degree of freedom and confidence levels ranging between 0.75 and 0.995.

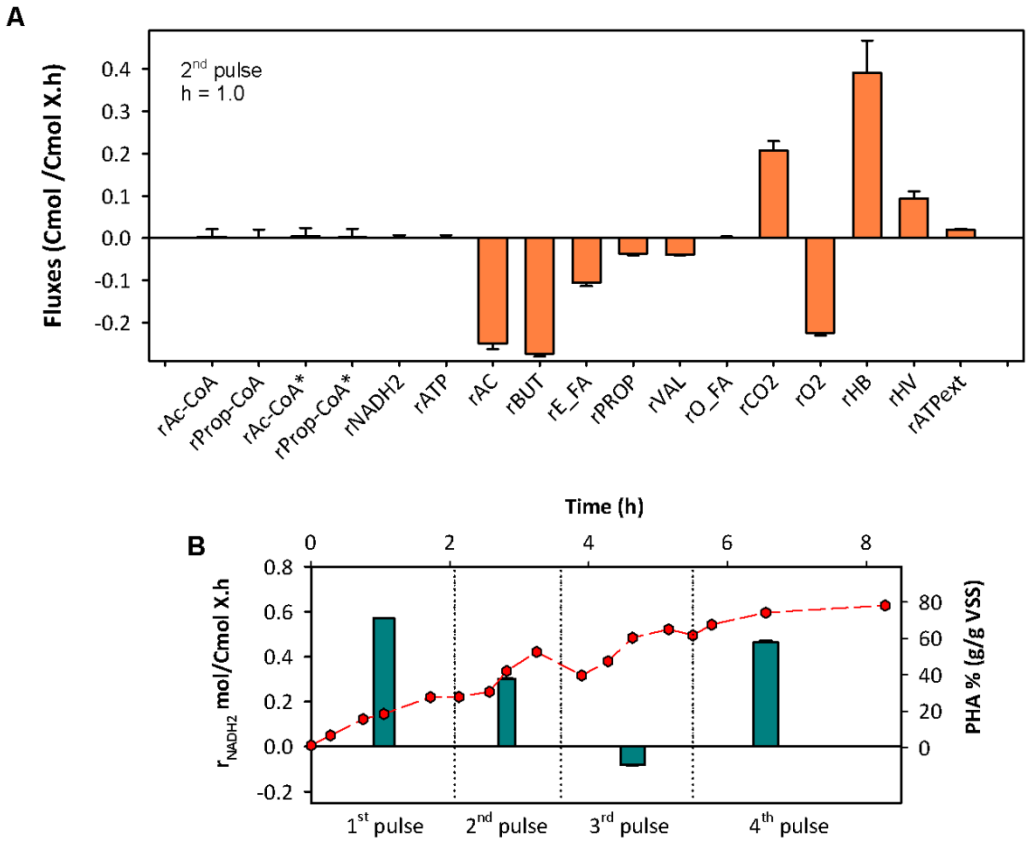


Figure 5.2: MFA results for FMIV batch accumulation experiment. A) Results for 2nd pulse of FMIV experiment when $NADH_2$ was balanced. B) Vertical bars show the results when unbalanced $NADH_2$ was assumed and its flux evolution trough the 4 pulses of FMIV batch experiment. Triangles and line show PHA content evolution over the 4 pulses of the same batch experiment.

Analysis of Table 5.1 shows that the MFA for the first pulse does not pass the consistency chi-square test in all experiments except SFI and FMIV. The same result was obtained for the last

pulses of some experiments (e.g. FMII, FMIV and SFI). As general trend it can be stated that the flux distributions in the first and last pulses are frequently inconsistent (with few exceptions), while in the middle pulses consistency is almost always obeyed (the only exception is FMII). The lack of consistency in the first and last pulses is most likely caused by the accumulation of intracellular metabolites. It is known that the $NADH_2$ and ATP pools may change significantly due to the transient carbon supply (Kessler and Witholt, 2001). After a long starvation phase the ATP and $NADH_2$ pools are low (Farabegoli et al., 2003). After feeding the first VFA pulse, the cells first replenish the $NADH_2$ and ATP pools. The increase of $NADH_2$ pool will eventually downregulate the TCA cycle, thereby inducing a carbon shift to PHA storage in order to consume the excess of $NADH_2$ (Kessler and Witholt, 2001). We have repeated the MFA calculations assuming unbalanced $NADH_2$ which translates into a determined non-redundant MFA system (Figure 5.2 B). These results show that with few exceptions the flux of $NADH_2$ is positive and higher in the first and last pulses, suggesting accumulation, while in the intermediate pulses $NADH_2$ steady-state condition is practically fulfilled. These results agree with the mechanism of TCA downregulation by $NADH_2$. Interestingly, these results also suggest a $NADH_2$ overflow upon PHA saturation as illustrated by the 4th pulse in Figure 5.2B. As PHA saturation is reached, consumption of $NADH_2$ decreased, $NADH_2$ accumulate and consequently, energetic metabolism is downregulated and carbon uptake cessed.

More details about the regulation effect of $NADH_2$ on carbon metabolism will be discussed in the sections below.

Table 5.1: Consistency index related to MFA calculations with one degree of freedom and values for χ^2 coefficient at several confidence levels considering 1 degree of freedom.

Pulse	Batch					
	FM II	FM III	FM IV	SF I	SF II	
1st	118.0	13.1	7.2	0.2	73.2	
2nd	42.2	0.7	1.0	0.3	6.1	
3rd	20.8	1.6	10.6	12.2	0.5	h values
4th		4.5	12.0	43.0	1.3	
5th		2.0		1.1		
Degrees of freedom	Confidence level (%)					
	75	90	97.5	99	99.5	
1	1.3	2.7	5.0	6.6	7.9	χ^2 values

5.4.2.2. Synthetic versus complex substrates

The use of complex carbon sources as substrate for PHA production was studied before by several authors (Beccari et al., 2009; Bengtsson et al., 2008; Mengmeng et al., 2009). These studies evaluated the effect of the substrate complexity in terms of kinetic parameters and system performance. Here we compare the batch experiments fed with synthetic VFA mixtures (SFI and SFII) mimicking the relative VFA composition of the fermented molasses experiment FMIII in order to investigate differences in MFDs. In SFI and SFII the maximum yield obtained was 0.78 C-mol PHA/C-mol VFA.

We restrict our analysis to the 2nd pulses of FMIII, SFI and SFII due to the consistency problems reported above. Figure 5.3A shows the normalized VFA uptake fluxes together with the respective total uptake in Cmmol DOC/Cmmol X.h (r_S), Figure 5.3B shows the normalized PHA and CO_2 production fluxes, and Figure 5.3C shows the normalized intracellular fluxes. To note that all fluxes were normalized by the total carbon uptake rate (r_S).

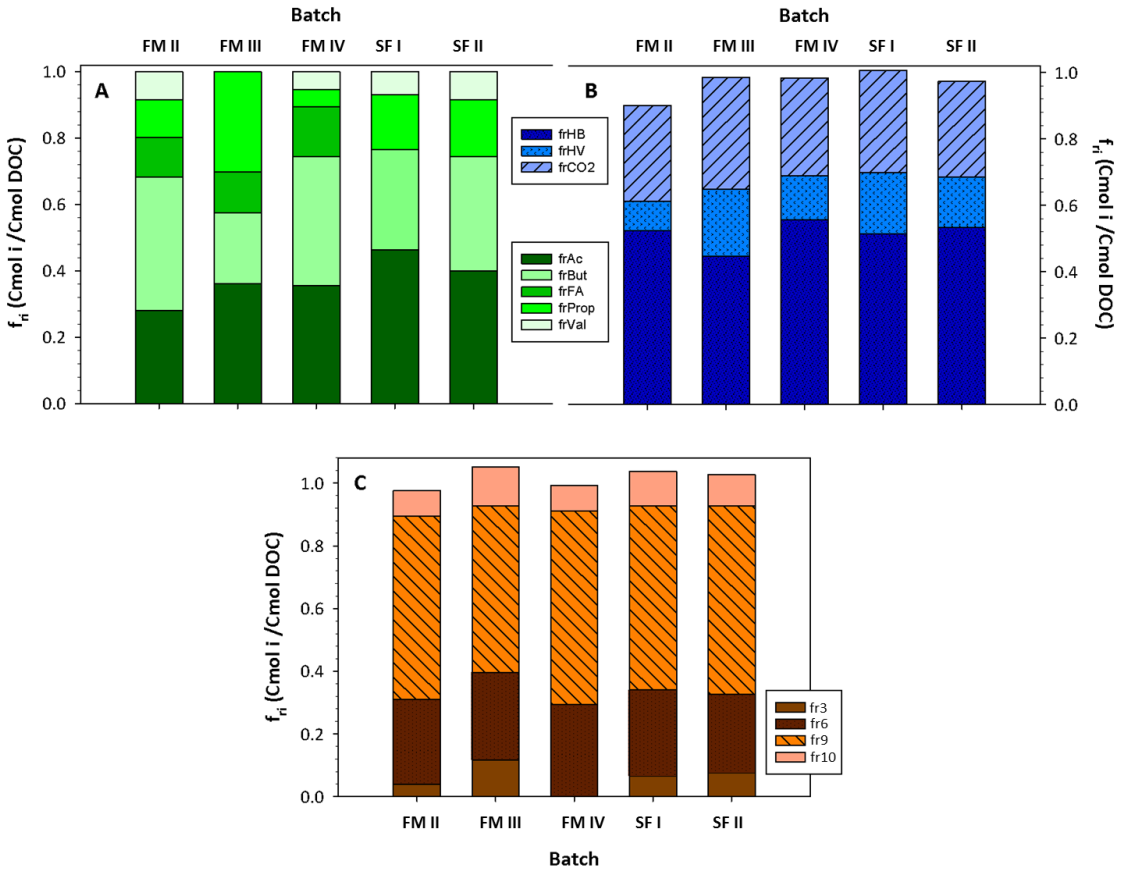


Figure 5.3: Metabolic fluxes (normalized between 0-1) calculated for the 2nd pulse of each batch accumulation experiment. Each flux is divided by the respective total uptake rate (r_S (Cmol DOC/Cmol X.h)), which are 0.21 ± 0.02 (FM II), 0.58 ± 0.03 (FM III), 0.70 ± 0.02 (FM IV), 0.43 ± 0.02 (SF I) and 0.35 ± 0.02 (SF II). A) Normalized uptake fluxes for individual substrates. B) Normalized production fluxes of HB, HV and CO_2. C) Normalized carbon related intracellular fluxes (r_3, r_6, r_9 and r_{10}).

The total uptake flux in FMIII (r_S = 0.58 ± 0.03 Cmmol DOC/Cmmol X.h) is 36% higher than in SFI (r_S = 0.43 ± 0.02 Cmmol DOC/Cmmol X.h) and 66% higher than SFII (r_S = 0.35 ± 0.03 Cmmol DOC/Cmmol X.h). Likewise the PHA production fluxes are 26% higher in FMIII (r_{PHA} = 0.38 ± 0.04 Cmmol HA/Cmmol X.h) than in SFI (r_{PHA} = 0.30 ± 0.02 Cmmol HA/Cmmol X.h) and 58% higher than in SFII (r_{PHA} = 0.24 ± 0.02 Cmmol HA/Cmmol X.h). These results are unexpected since the amount of carbon supplied in the 2nd pulses of SFI and SFII was higher than in FMIII (Table 3.2). Despite the big differences in total rates, only moderate differences are observed in the normalized MFD (Figure 5.3 A, B and C), probably caused by small variations in VFA composition among batch experiments (51:24:9:0:16, 60:16:20:5:0 and 61:15:20:4:0 Ac:Prop:But:Val:FA for FMIII, SFI and SFII, respectively).

These results illustrate the effect of the complex substrate composition on the metabolic fluxes distribution. The main difference between these experiments is the unidentified FA (16-25% of carbon), present in FMIII but absent in SFI and SFII. These results show that the MMC developed a preference for the substrate used in the enrichment stage, which naturally includes the unknown fraction FA with a relatively high uptake flux (see flux f_{rFA} in Figure 5.3A). The use of synthetic VFA mixtures mimicking the fermented molasses did not produced the same storage efficiency, which further highlights the importance of the unknown FA fraction in the overall VFA uptake regulation.

5.4.2.3. Effect of culture enrichment time

Batch experiments FMII and FMIV were carried out using similar fermented molasses feedstock composition (41:18:25:11:4 and 41:30:20:6:4 Ac:But:FA:Prop:Val, respectively, in FMII and FMIV and, in both, no ammonia was detected). The difference lies on the MMC inoculum that was collected from the enrichment SBR 115 days apart. Thus the observed differences in PHA accumulation performance (saturation PHA content increased from 58 to 75%) can be attributed mainly to the time of enrichment.

In order to quantitatively investigate the effect of enrichment time in metabolic fluxes, MFA calculations were performed for the 2nd pulse of FMII and FMIV experiments. Not only the VFA composition but also the DOC and biomass concentrations are comparable (see Table 3.2). The differences in MFDs, normalized by total carbon uptake rate (r_S), between these two experiments are shown in Figure 5.3.

As first striking result, it can be seen that the culture selected over the 115 days of culture enrichment is able to process higher amount of carbon in both substrate uptake and PHA

production fluxes (which globally increased, respectively, by 70% and 73% from FMII to FMIV). On the other hand, it is interesting to note that the normalized intracellular carbon fluxes distribution does not change very much as a consequence of culture enrichment. There is however some differences that calls for a more careful interpretation. One of them is that the uptake of VFA with even number of carbon atoms increased by 10% whiles those with odd number of carbon atoms decreased by 90% from FMII to FMIV. The shift from odd to even carbon number VFA uptake is accompanied by the almost complete cessation of acetyl-CoA production from propionyl-CoA precursors with concomitant loss of a CO_2 molecule (r_3 = 0 Cmol/Cmol X.h in FMIV). In the more enriched culture, VFA with odd number of carbons were almost entirely used for HV synthesis through formation of propionyl-CoA precursors combined with acetyl-CoA molecules synthesized from substrates with even number of carbons. It is interesting to note that despite the shift from odd to even VFA uptake the HV fraction in the final polymer increased due to the more efficient use of propionyl-CoA for HV synthesis.

It is also worthwhile to note that percentage of carbon driven to TCA cycle presents a slight increase of 7% over enrichment period, which is counterintuitive since more carbon spent for energy generation also means less carbon stored as PHA. However, this increase in TCA cycle can be explained by the higher ATP that is consumed in the uptake of even carbon number VFA (acetate and butyrate) in relation to that consumed in the uptake of odd VFA. In this case there seems to be a trade-off between the percentage of carbon lost for energy generation and the magnitude of total carbon stored as PHA, with the latter factor prevailing over the former.

5.4.3. Flux balance analysis

We have implemented an FBA program to predict the PHA production flux and the HB:HV composition for a given set of input VFA uptake rates. As described in the material and methods section, this problem is undetermined and cannot be calculated by MFA. It can however be calculated by FBA provided that a meaningful metabolic objective function is known. Several studies have demonstrated the downregulation effect of TCA cycle on PHA production pathway (Henderson and Jones, 1997; Park and Lee, 1996). Given this evidence and also the results above of the TCA cycle regulation by $NADH_2$ and its effect on PHA accumulation we have implemented a FBA that minimizes the TCA flux (R_4) in the feast phase of MMC cultures subjected to the feast and famine enrichment regimen. Additionally, we have fixed the flux ratio between r_3 and r_8 at 0.67. This ratio reflects the proportion of acetyl-coA* and propionyl-coA* required to produce a HV monomer in case that only substrates with odd number of carbon atoms are available.

Figures 5.4 A and B compares the HB and HV monomers production fluxes, calculated as described in chapter 3, versus the predicted by FBA for all pulses that pass consistency test (see section 5.4.2.1). For both monomers, the FBA predictions presents a slight dispersion of data since the R^2 is, respectively, 0.82 and 0.66 for HB and HV fluxes. Despite this result, a good agreement between FBA predictions and MFA results was achieved since the slope of the fitting is very close to 1 for both monomers. Moreover, if one consider the total PHA production fluxes (Figure 5.4 C), the agreement between predicted versus experimental is even closer to 1 (slope = 1.02) and the dispersion effect is slightly minimized (R^2=0.84).

These results demonstrate the minimization of TCA cycle as an appropriate cellular objective function for a MMC enriched in PHA organisms by the feast and famine regimen.

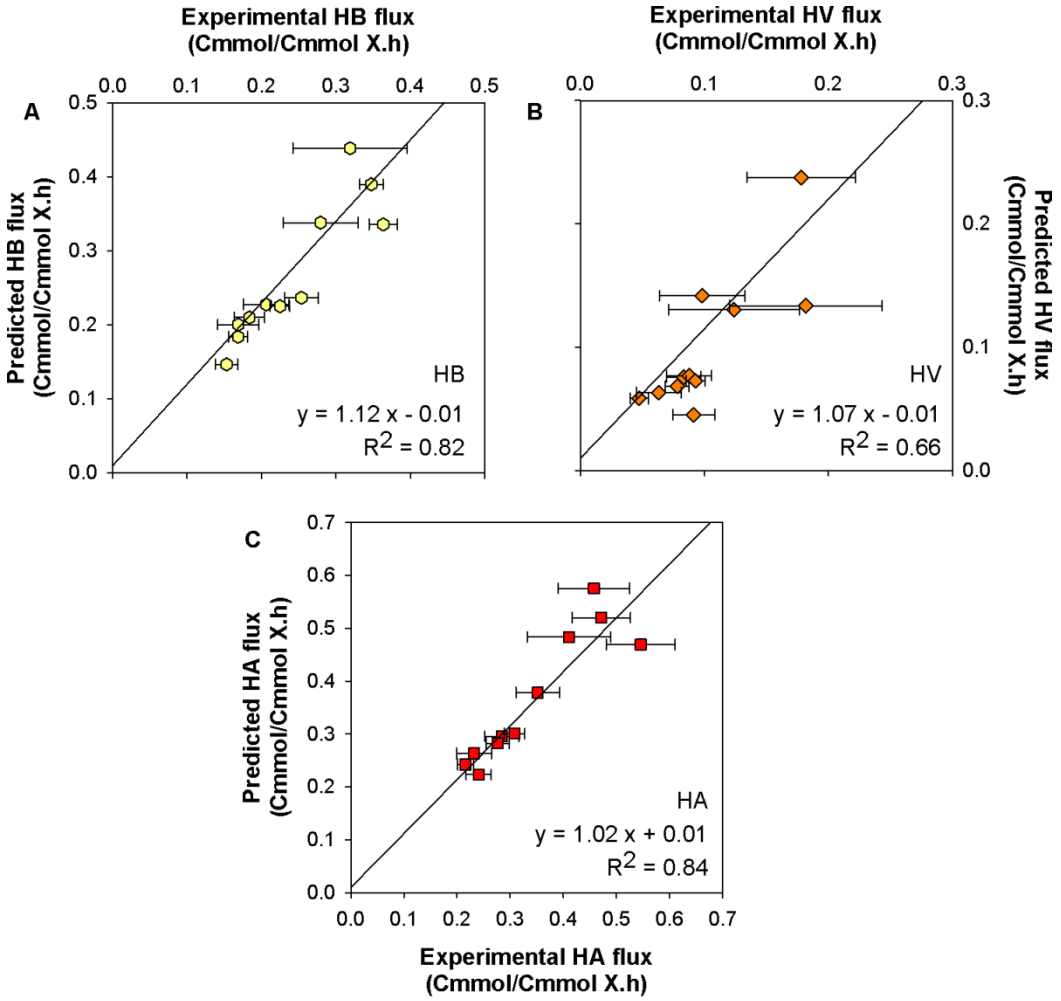


Figure 5.4: FBA results for prediction of PHA production using only substrates rates calculated by MFA as inputs. A) Results for prediction of HB monomer production. B) Results for prediction of HV monomer production. C) Results for the sum of HB and HV monomers production.

5.5. Conclusions

In this work we have investigated PHA accumulation from complex fermented sugar cane molasses using a lumped MMC metabolic model that describes PHA storage from arbitrary mixtures of volatile fatty acids. The MMC was subjected to the feast and famine feeding regimen using fermented sugar cane molasses as substrate. MMC samples were collected at the end of the famine phase and then used to inoculate a batch reactor to study the PHA storage efficiency by feeding with consecutive pulses of either fermented sugar cane molasses or synthetic VFA mixtures. At the aid of the metabolic model, the following main conclusions could be withdrawn:

- In the first pulse, unbalanced $NADH_2$ is frequently verified. This can be explained by the metabolic state of cells at end of famine phase (low $NADH_2$ pool). In the first pulse the $NADH_2$ increases and at some point the high $NADH_2$ intracellular level downregulates the TCA cycle and the substrate is preferentially driven towards PHA storage (in the 2^{nd} pulse and further).
    - Also in the last (4^{th} or 5^{th}) pulses unbalanced $NADH_2$ is frequently verified, which is coincident with PHA storage cessation but not necessarily with PHA storage saturation.
    - MFDs were calculated for both complex and synthetic VFA mixtures experiments. The carbon distribution is not much different when comparing both cases. However, the metabolic rates are much higher for the complex fermented sugar cane molasses case. Possibly the fermented sugar cane molasses, which was the substrate used in the MMC selection stage, contain compounds involved in the regulation of the metabolism that justifies the observed differences in the MFDs.
    - MFDs calculations revealed that the substrate uptake and PHA storage efficiency increase with time of enrichment. This gain in efficiency can be partially attributed to a shift in the uptake of VFA with even number of carbons towards the preferable uptake of VFA with odd number of carbons and the reduction of the decarboxilation flux (r_3), which generically improves metabolic efficiency.

Finally it was shown that it is possible to predict PHA storage fluxes from measurements of VFA fluxes by minimizing the carbon processes in the TCA cycle. An accurate prediction of PHA fluxes and HB:HV composition was obtained by flux balance analysis that minimizes the TCA cycle. This computational result essentially confirms that the feast and famine feeding regimen results in an optimised MMC that maximises carbon usage for PHA storage.

6 Segregated flux balance analysis

In this study we developed a segregated flux balance analysis (FBA) method to calculate metabolic flux distributions of the individual populations present in a mixed microbial culture (MMC). Population specific flux data constraints were derived from the raw data typically obtained by the Fluorescence in situ hybridization (FISH) and microautoradiography (MAR)-FISH techniques. This method was applied to study the metabolic heterogeneity of a MMC that produces polyhydroxyalkanoates (PHA) from fermented sugar cane molasses. Three populations were identified by FISH, namely Paracoccus sp, Thauera sp and Azoarcus sp. The segregated FBA method predicts a flux distribution for each of the identified populations. The method is shown to predict with high accuracy the average PHA storage flux and the respective monomeric composition for 16 independent experiments. Moreover, flux predictions by segregated FBA were slightly better than those obtained by nonsegregated FBA, and also highly concordant with metabolic flux analysis (MFA) estimated fluxes. The segregated FBA method can be of high value to assess metabolic heterogeneity in MMC systems and to derive more efficient eco-engineering strategies. For the case of PHA-producing MMC considered in this work, it becomes apparent that the PHA average monomeric composition might be controlled not only by the volatile fatty acids (VFA) feeding profile but also by the population composition present in the MMC.

The contents of this chapter were adapted from the publication: Pardelha F, Albuquerque MGE, Carvalho G, Reis MAM, Dias JML, Oliveira R. 2013. Segregated flux balance analysis constrained by population structure/function data: the case of PHA production by mixed microbial cultures. Biotechnology and Bioengineering. DOI: 10.1002/bit.24894[1]

6.1. Introduction

Mixed microbial cultures (MMC) applications are currently evolving from the traditional waste treatment technologies to novel biological production platforms of value added products from waste materials. In this respect, MMC have several advantages over pure cultures, such as the ability to process complex waste streams, the ability to adapt to changes in substrate/operating conditions, nonsterile operation and very robust continuous process operation (Kleerebezem and van Loosdrecht, 2007).

In recent years, new experimental techniques and design tools have been developed in order to improve knowledge and to derive more effective eco-engineering strategies for MMC systems. Fluorescent in situ hybridization (FISH) combined with microautoradiography (MAR) is a technique that can be used for structure-function studies in microbial ecology (Lee et al., 1999). This experimental technique links FISH, used to identify individual species within a MMC through fluorescently labelled ribosomal RNA-targeted oligonucleotide probes (DeLong et al., 1989), with MAR to detect substrate specific consumption using radioactively labelled substrates. MAR-FISH is becoming a widely used tool to study the ecophysiology of microbial communities (Albuquerque et al., 2013; Hagman et al., 2008; Nielsen et al., 1999). The concurrent application of MAR and FISH techniques to polyhydroxyalkanoates (PHA) producing MMCs was reported, for the first time, by Albuquerque et al. (2013). This is the first experimental evidence that different populations within a PHA-producing MMC have different substrate uptake preferences. However, it remains to be understood how those different substrate uptake preferences translate in heterogeneous PHA production metabolism.

In this study, the main focus is the development of a modelling tool to analyze the metabolic heterogeneity in MMC cultures supported by FISH and MAR-FISH data. More specifically, we focus on the well known PHA production process by MMC subjected to the feast and famine eco-engineering strategy (Albuquerque et al., 2007; Beccari et al., 1998). In the last decade, several metabolic models have been proposed that describe PHA accumulation by MMC (Beun et al., 2000b; Dias et al., 2005, 2008; Jiang et al., 2011b; Johnson et al., 2009b). All those metabolic models have in common I) the use of lumped metabolic reactions, II) the assumption of an average metabolism representing all populations in the MMC and III) the use of a MMC fed with synthetic substrate(s). Although I) and II) have been generally accepted in the MMC scientific community (Beun et al., 2000b; Dias et al., 2005, 2008; Jiang et al., 2011b; Johnson et al., 2009b), it is clear that such modelling approaches fail to discriminate and characterize the individual populations in the MMC.

In a previous study, Pardelha et al. (2012 - chapter 5) developed a lumped flux balance analysis (FBA) with the ability to predict PHA production rate and respective monomeric composition from the rates of volatile fatty acids (VFA) uptake. FBA is a constraint-based modelling approach commonly used to model underdetermined metabolic networks of single microorganisms (Orth et al., 2010). The method applies optimization methods to minimize/maximize a given metabolic objective, which must be defined a priori, under the constraint of reactions stoichiometry, reactions irreversibility and experimental flux data. Here we extend the FBA method to a segregated FBA that considers different populations through the inclusion of additional constraints imposed by quantitative FISH and qualitative MAR-FISH data. The experimental data were obtained from a MMC PHA production process using fermented sugar cane molasses (FM) as feedstock (Albuquerque et al., 2010b, 2013).

6.2. Materials and methods

The description of all material and methods relevant for this work is presented on chapter 3 of this Ph.D. thesis. The batch experiments used in the present chapter were FMI, FMII, FM III, FMIV and FM V for fermented molasses.

6.2.1. Experimental data used for model development

6.2.1.1. Experimental flux data

To determine experimental fluxes, a set of five PHA production batch experiments were performed using clarified fermented molasses as feed solution (reactor 3 of the PHA production process described in chapter 3), at different stages of SBR enrichment along 27 months of operation (reactor 2). The same approach described in Chapter 3 was adopted to calculate uptake/production fluxes and respective standard deviations. Briefly, each batch experiment was segmented in time intervals according to the pulse-wise VFA feeding implemented. All concentrations were divided by the active biomass concentration, which was approximately constant within each batch experiment given the absence of a nitrogen source. The slope and standard deviation of the linear fit of concentrations divided by biomass along time provided the specific rate value and respective standard deviation. This procedure resulted in the dataset of 16 measured rate vectors shown in Table 3.3. To note that 88% of the linear regression correlation coefficients, R^2, were higher than 0.90 and in all cases higher than 0.73 (results not shown). For those cases where the linear fit is less reliable, the respective standard deviation is higher, which is then taken into consideration in the FBA modelling method described below.

6.2.1.2. Microbial population quantification data (FISH data)

Population composition normally converges to an equilibrium for constant SRT, HRT and organic load and composition (Jiang et al., 2011b; Johnson et al., 2009a). In order to identify and quantify the microbial populations present in the MMC, biomass samples were taken from the enrichment SBR (reactor 2) and immediately fixed in 4% paraformaldehyde (PFA) for FISH analysis. Biomass fixation and FISH analysis were performed according to the methodology proposed by Amann (1995). The fluorescently labeled oligonucleotide probes used were as follows: EUBmix, for all Bacteria (a mixture of probes EUB338 (Amann et al., 1990), EUB338-II and EUB338-III (Daims et al., 1999), Azo644 for most members of the Azoarcus cluster (Hess et al., 1997), THAU832 for Thauera (Loy et al., 2005), PAR651 for Paracoccus (Neef et al., 1996). Hybridized samples were viewed with a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM). FISH quantification of each specific probe (Cy3-labeled) in respect to all Bacteria (Cy5-labeled) was carried out by image analysis of 30 images of each sample using the Daime software (Daims et al., 2006), which determines the biovolume fraction of the specifically labeled target population relative to the biovolume of the total bacteria. The standard error of the mean (SEM) was calculated as the standard deviation divided by the square root of the number of images. Table 6.1 compiles the quantification results for the 3 populations detected at the different studied periods.

Table 6.1: Microbial population quantification results for the different periods of batch accumulation experiments.

	Enrichment time (days)	f_{Azo}	f_{Thau}	f_{Par}
FMI	68	0.08 (0.01)	0.87 (0.01)	0.012 (0.002)
FMII	247	0.006 (0.002)	0.15 (0.01)	0.47 (0.03)
FMIII	324	0.12 (0.01)	0.67 (0.02)	0.025 (0.003)
FMIV	364	0.65 (0.01)	0.26 (0.01)	0.007 (0.001)
FMV	860	0.26 (0.01)	0.19 (0.01)	0.51 (0.01)

The fractions of each population were calculated based on biovolume fraction relative to the biovolume of the total bacteria. The standard error of the mean is given in brackets.

6.2.1.3. Microbial population characterization data (MAR-FISH data)

The PHA-producing MMC studied in this work was previously investigated in respect to the substrate uptake preferences by each of the dominant populations present in the enrichment

through MAR-FISH (Albuquerque et al., 2013). The authors performed two types of experiments (with single and multiple substrates), where the one relevant to the present study was the determination of uptake of a labeled substrate in the presence of other (unlabeled) substrates. The substrates investigated were acetate, propionate, butyrate and valerate (the main substrates present in fermented sugar cane molasses).

The MAR-FISH results presented by Albuquerque et al. (2013) consist of four semi-quantitative classifications (++ strong uptake; + medium uptake; (+) low uptake; - very low uptake) according to the density of the MAR signal. The conversion of this qualitative information to quantitative data was based in the assignment of an interval of fraction of measured overall uptake rate for each of the symbolic classifications: ++ → [1.0, 0.75]; + → [0.75, 0.50]; (+) → [0.50, 0.25] and - → [0.25, 0.00]. Table 6.2 presents the results obtained by Albuquerque et al. (2013) along with the respective conversion made in the present study.

Table 6.2: Results of MAR-FISH (Adaption from Albuquerque et al. 2013) and respective numerical intervals assumed in this work.

Substrate labeled in the mixture	*Azoarcus*	*Thauera*	*Paracoccus*
^{3}H-Acetate	++ 1.0 – 0.75	- 0.25 – 0.0	+ 0.75 – 0.50
^{14}C-Propionate	(+) 0.50 – 0.25	(+) 0.50 – 0.25	++ 1.0 – 0.75
^{14}C-Butyrate	+ 0.75 – 0.50	++ 1.0 – 0.75	++ 1.0 – 0.75
^{14}C-Valerate	(+) 0.50 – 0.25	(+) 0.50 – 0.25	++ 1.0 – 0.75

Symbols represent the qualitative results obtained by Albuquerque et al. 2013 for substrate uptake in a multiple substrate environment. The numerical intervals presented below symbols represent the corresponding fraction of overall uptake rate.

++ strong uptake; + medium uptake; (+) low uptake; - very low uptake

6.2.2. Segregated flux balance analysis

The methodology chosen to develop the metabolic model in this study was FBA. The implementation of FBA undergoes four main steps: I) state the metabolic network of the target organism(s); II) define constraints, which include steady state material balances over intracellular metabolites, irreversibility of metabolic reactions, and constraints arising from problem specific measured variables; III) define an objective function and IV) apply an optimization method that maximizes/minimizes the objective function (a theoretical metabolic flux distribution) under the defined set of constraints (Raman and Chandra, 2009). The advantage of FBA relatively to other standard metabolic modelling methods is that it can be applied to underdetermined systems, that is, systems for which the number of unknown fluxes is higher than the number of equality constraints. Indeed, MMC metabolic modelling problems tend to be expressed by largely underdetermined algebraic systems of equations because each of the populations are themselves expressed by underdetermined systems where the number of intracellular metabolites is much lower than the number of reactions.

In a previous work we have studied nonsegregated FBA of PHA accumulation by MMC (Pardelha et al., 2012 - Chapter 5). A lumped metabolic network was adopted comprising 14 metabolic reactions (Tables 4.1 and 4.2) and 17 metabolites, from which 6 are intracellular. This metabolic network forms a system of algebraic equations with 6 equations (material balance equations over intracellular metabolites) and 14 unknown variables (metabolic fluxes). In Pardelha et al. (2012 – Chapter 5), metabolic flux analysis was applied to calculate 5 unknown intracellular fluxes (v_3, v_4, v_6, v_7, v_8) from measurements of the remaining 9 (extracellular) fluxes, which resulted in an over-determined algebraic system with 1 degree of freedom. An important conclusion from this study was that the metabolic network stoichiometry (Tables 4.1 and 4.2) was consistent with the measured flux data as demonstrated by the well-known consistency index test (Pardelha et al., 2012 - Chapter 5). Moreover, a non-segregated FBA method was implemented, using the previously validated metabolic network (Tables 4.1 and 4.2). One of the main results of the non-segregated FBA study was that the minimization of the catabolic flux (flux v_4 in the metabolic network, see Table 4.1) under the constraint of measured VFA uptake fluxes successfully predicts the PHA accumulation flux and the respective HB:HV monomeric composition. We now aim to extend the previous FBA formulation to the segregated case by considering the metabolism of the 3 distinct populations identified by FISH (Table 6.1). It was considered that the metabolic network is the same for the 3 populations while the metabolic fluxes may vary. Furthermore, given the success of the lumped FBA model previously reported (Pardelha et al., 2012 - Chapter 5) we consider that the metabolic objective, i.e. minimization of

TCA activity, is the same for all the 3 populations identified by FISH. As such the FBA optimization problem is stated as follows:

$$\min_{\mathbf{v}^{(1)},\mathbf{v}^{(2)}\mathbf{v}^{(3)}} \quad Z = v_6^{(1)} + v_6^{(2)} + v_6^{(3)} \tag{6.1}$$

The superscript index denotes population, that is, j=1 (*Azoarcus*), j=2 (*Thauera*) and j=3 (*Paracoccus*). The degrees of freedom are the metabolic flux vectores, $v^{(j)}$, of each population j. The number of degrees of freedom are thus 14 (fluxes per population) × 3 (populations) = 42. Z is the objective function and $v_6^{(j)}$ the catabolic flux of population j.

The 6 (intracellular metabolites) × 3 (populations) = 18 steady-state material balance equations define 18 equality constraints as follows:

$$0 = \underbrace{\begin{bmatrix} 1 & -1 & 0 & 0 & -1 & 0 & 0 & 0 \\ -1.5 & 0 & 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & -1 & -0.4 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & -0.6 \\ 1.5 & 2 & 0 & -1 & -0.25 & -0.17 & 0 & 0 \\ 0 & 0.5 & -1 & 1 & 0 & 0 & 0 & 0 \end{bmatrix}}_{\mathbf{A}_n} \cdot \mathbf{v}_n{}^{(j)} + \underbrace{\begin{bmatrix} 1 & 1 & 1 & 0 & 0.4 & 0 \\ 0 & 0 & 0 & 1 & 0.6 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0.5 & 0 & 0 & 0.4 & 0 \\ -1 & -0.5 & -1 & -0.67 & -0.4 & -0.67 \end{bmatrix}}_{\mathbf{A}_b} \cdot \mathbf{v}_b{}^{(j)} \qquad \text{j=1,2,3} \tag{6.2}$$

with $\mathbf{v}_n^{(j)} = \left\{v_l^{(j)}\right\}^T$ the unknown flux vector comprising the metabolic fluxes l=3,6,7,8,9,10,HB,HV and $\mathbf{v}_b^{(j)} = \left\{v_k^{(j)}\right\}^T$ the measured flux vector comprising fluxes k=Ac,But,E_FA,Prop,Val,O_FA (see Tables 4.1 and 4.2 and Chapter 5) for details about the metabolic network).

In Pardelha et al. (2012 - Chapter 5) a regulatory constraint was identified imposing that the split of propionyl-CoA flux into v_3 and v_8 is regulated in such a way that the stoichiometry of the condensation of acetyl-CoA* with propionyl-CoA* into HV is obeyed. This gives rise to 3 additional equality constraints:

$$v_3^{(j)} - 0.67 v_{10}^{(j)} = 0 \qquad \text{j=1,2,3} \tag{6.3}$$

Irreversible metabolic reactions define 42 additional inequality constraints

$$v^{(j)}, v^{(j)}, \ldots \geq 0 \qquad \text{j=1,2,3} \tag{6.4}$$

The weighted sum of population metabolic fluxes by the respective biomass fraction must match the overall measured fluxes within the error interval. This results in the following set of 18 inequality constraints:

$$\mathbf{b} - \boldsymbol{\sigma}_b \leq \sum_j \mathbf{v}_b^{(j)} f_X^{(j)} \leq \mathbf{b} + \boldsymbol{\sigma}_b \qquad \text{j=1,2,3} \tag{6.5}$$

with b=[v_{Ac}, v_{But}, v_{E_FA}, v_{Prop}, v_{Val}, v_{O_FA}]^T the measured overall flux vector (see Table 3.3) and $f_X^{(j)}$ the biomass mass fraction of population j calculated as follows:

$$f_X^{(j)} = \frac{X^{(j)}}{\sum_j X^{(j)}} \tag{6.6}$$

Finally, the population substrate uptake preferences determined by MAR-FISH (Table 6.2) resulted in additional 18 inequality constraints:

$$\alpha_k^{(j)} b_k \le \sum_j v_k^{(j)} f_X^{(j)} \le \beta_k^{(j)} b_k \qquad \text{j=1,2,3} \qquad \text{k=Ac,But,E\_FA,Prop,Val,O\_FA} \tag{6.7}$$

with $\alpha_k^{(j)}$ and $\beta_k^{(j)}$ the lower and upper bounds of the MAR-FISH classification taken from Table 6.2. For example Table 6.2 shows that the population j=1 has a strong acetate uptake, which corresponds to the interval [1.0; 0.75]. In conformity $\alpha_{Ac}^{(1)}$ is set to 0.75 and $\beta_{Ac}^{(1)}$ is set to 1.0.

To solve this optimization problem a MATLAB code was implemented based in the constrained nonlinear programming method implemented in function *fmincon*.

6.2.3. Metabolic flux analysis

Standard lumped metabolic flux analysis (MFA) was also implemented in order to compare with FBA predictions. Unlike FBA, MFA does not require an objective function because it can only be applied to determined systems. The metabolic network used for FBA was also used for MFA, as it is assumed to represent an “average” MMC. The idea is to use all available measured fluxes to calculate the unknown intracellular fluxes, and then to compare the MFA results with the FBA results. As such, the unknown, N, and measured, B, flux partitions were defined as follows

$$0 = \underbrace{\begin{bmatrix} 1 & -1 & 0 & -1 & 0 \\ -1.5 & 0 & 0 & 0 & -1 \\ 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 1.5 & 2 & -1 & -0.25 & -0.17 \\ 0 & 0.5 & 1 & 0 & 0 \end{bmatrix}}_{\mathbf{A}_n} \cdot \mathbf{v}_n + \underbrace{\begin{bmatrix} 1 & 1 & 1 & 0 & 0.4 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0.6 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & -1 & -0.4 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & -0.6 & 0 \\ 0 & 0.5 & 0 & 0 & 0.4 & 0 & 0 & 0 & 0 \\ -1 & -0.5 & -1 & -0.67 & -0.4 & -0.67 & 0 & 0 & -1 \end{bmatrix}}_{\mathbf{A}_b} \cdot \mathbf{v}_b \tag{6.8}$$

with $\mathbf{v_n}$=[v_3,v_4,v_6,v_7,v_8]^T and $\mathbf{v_b}$=[v_{Ac}, v_{But}, v_{E_FA}, v_{Prop}, v_{Val}, v_{O_FA}, v_{HB}, v_{HV}, ,v_5]^T. This gives rise to an overdetermined system of algebraic equations with 5 unknown fluxes (since v_5 stands for maintenance and is fixed (v_7 = m_{ATP} = 0.02 mol ATP/(C-mol h))) that can be solved as follows:

$$\mathbf{v}_n = -\mathbf{A}_n^{\#} \cdot \mathbf{A}_b \cdot \mathbf{v}_b \tag{6.9}$$

where $\mathbf{A}_n^{\#}$ is the Moore-Penrose pseudo-inverse matrix of $\mathbf{A}_n$. Also the Chi-squared (χ^2) statistical test was implemented to validate the consistency between assumed metabolic constraints and measured flux vectors corrupted by experimental error. For more details see Pardelha et al. (2012 - Chapter 5).

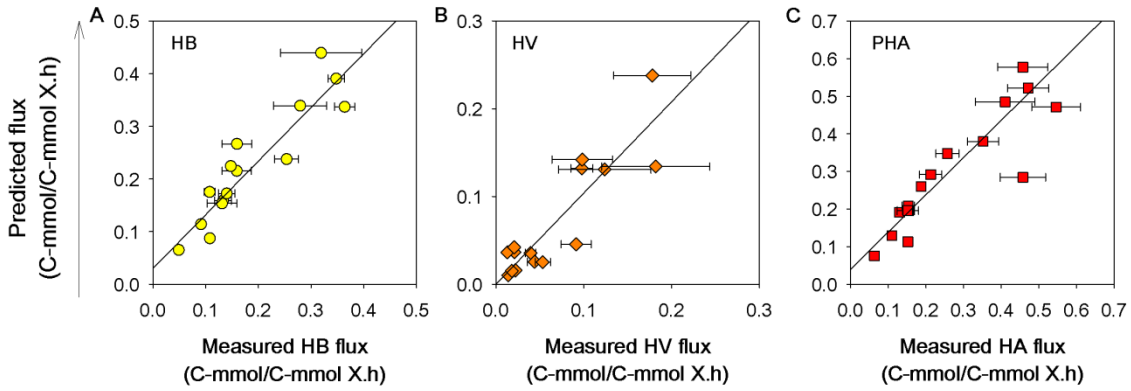
6.3. Results and discussion

6.3.1. Prediction of PHA production fluxes

FBA based techniques can be applied to undetermined systems as mentioned previously. However, if experimental fluxes are used as constraints to FBA calculations, more realistic FBA predictions can be obtained. Figure 6.1 A and B compare the experimental fluxes of HB and HV, respectively, with the corresponding FBA predictions. Since the segregated FBA calculates a flux vector for each of the populations, the respective weighted sum must be calculated in order to compare with the overall experimental value:

$$v_i = \sum_j v_i^{(j)} f_X^{(j)} \tag{6.10}$$

with i=HB,HV. Regression coefficients (R^2) were 0.85 and 0.79 for HB and HV, respectively, with both slopes very close to 1 (HB: 1.02 and HV: 1.04). These results indicate that segregated FBA model was able to consistently predict global HB and global HV production along different steady states periods of culture enrichment (with a different microbial community structure), as well as different feeding scenarios (relative abundance of each VFA's present in FM vary between batch experiments). When the global PHA fluxes are compared, experimental versus predicted (Figure 6.1 C), both regression coefficient and slope are even better (R^2 = 0.90 and slope = 0.99) than for the individual monomers case. Slope values close to 1.0 indicate that the model is unbiased and that prediction error variance is mainly due to random experimental error. Overall, it can be stated that the proposed segregated FBA model can consistently predict PHA fluxes and respective monomeric composition.



D

	Slope			Correlation coefficient (R^2)		
	HB	HV	PHA	HB	HV	PHA
Nonsegregated FBA (Pardelha et al. 2012)	1.12	1.07	1.02	0.82	0.66	0.81
Segregated FBA (this work)	1.02	1.04	0.99	0.85	0.79	0.9

Figure 6.1: Predicted over measured PHA production fluxes by the segregated FBA. Predicted fluxes are average values of the 3 populations. A) HB monomer production flux. B) HV monomer production flux. C) PHA production flux (sum of HB and HV predictions). D) Slopes and correlation coefficients for segregated FBA (this work) and nonsegregated FBA (Pardelha et al., 2012 – Chapter 5).

Pardelha et al. (2012 - Chapter 5) presented a nonsegregated FBA model for the same process (metabolic network), with the same objective function (catabolic flux minimization), obeying the same constraints (experimental substrate uptake fluxes and v_3/v_8 ratio) except for the FISH and MAR-FISH data derived constraints. Figure (1D) compares the prediction power between the segregated FBA model and the previously published nonsegregated FBA. From this comparison it can be observed that a slight improvement is obtained with the segregated FBA model in relation to the nonsegregated case reflected in slopes closer to 1.0 and higher correlation coefficients. The incorporation of FISH and MAR-FISH information thus introduced a small improvement in the prediction of average PHA production and monomeric composition.

6.3.2. Prediction of overall metabolic fingerprint

Figure 6.2 compares the segregated FBA predicted fluxes with the MFA estimated fluxes. Again, since the segregated FBA predicts population specific metabolic fluxes, Eq. 6.10 was applied to calculate the MMC average fluxes in order to be able to compare with the MFA results. Figure 6.2A presents the results of all fluxes for the 2nd pulse of the FM IV batch experiment. Figure 6.2B shows all fluxes of all experiments in a single linear regression plot excluding the measured input fluxes, namely v_{Ac}, v_{But}, v_{Prop}, v_{Val} and v_{FA}. It can be observed that the FBA predicted fluxes

are in close agreement with the MFA estimated fluxes. The correlation coefficient in Figure 6.2B is 0.99 and the slope is 1.01. These results support the FBA assumptions regarding the objective function and the constraints adopted.

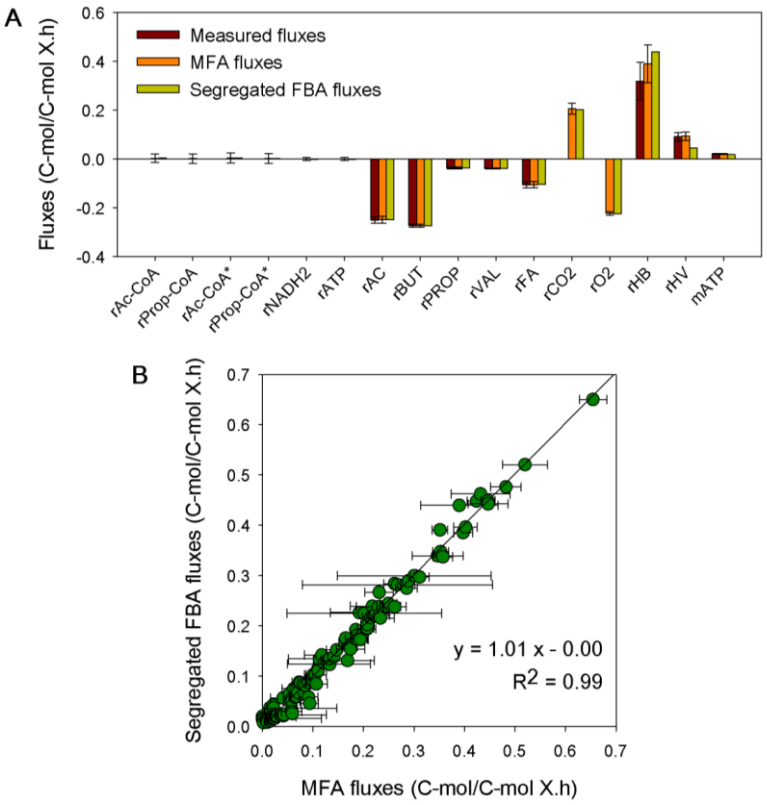


Figure 6.2: Comparison between segregated FBA, MFA and measured fluxes. Segregated FBA predicted fluxes are average values of the 3 populations. A) Metabolic fluxes for the 2nd pulse of FM IV batch experiment. B) Correlation between MFA and segregated FBA fluxes excluding the measured input fluxes (v_{Ac}, v_{But}, V_{Prop}, v_{Val} and v_{FA}) for all experiments.

6.3.3. Metabolic variability among microbial populations

The composition of the microbial population as determined by MAR-FISH shows considerable variations over time (Table 6.1). The organic loading rate to the SBR was stable, however the feedstock, produced in the upstream acidogenic reactor, showed fluctuations in the VFA composition distribution (Table 6.3). The varying VFA composition may induce population dynamics due to substrate uptake preferences by different species as previously analyzed by Albuquerque et al. (2013). The segregated FBA model developed in the present work does not provide a mean to predict population dynamics. It can be rather used as a tool to analyze the metabolic heterogeneity among the distinct microorganisms in a MMC. Figure 6.3 shows the metabolic flux distribution (MFD) calculated for the 3 populations for the 2nd pulse of FM IV batch

experiment (stacked bars in Figure 6.3). From these data it can be highlighted an overall higher substrate uptake rate by *Azoarcus* in comparison to *Paracoccus*, which in turn is higher than *Thauera.* This is reflected in the fluxes of the remaining reactions, except in HV production flux, resulting in higher HB production from *Azoarcus* than the other two bacterial groups (Figure 6.3). The exception of HV production flux (higher for *Paracoccus*) reflects the preference of *Paracoccus* for substrates with odd number of carbons (propionate and valerate). Figure 6.4 shows the distribution of VFA uptake flux (sum of VFA uptake rates) among the 3 populations for all the experiments. Comparing Figure 6.4 and Table 6.1, interestingly, notes that the overall VFA uptake rates are not always concordant with the respective population. For example, *Thauera* was always responsible for lower VFA uptake rate than the other 2 populations, even when its biomass fraction is the highest.

The variability in the metabolic activity among the three populations is also reflected in the copolymer composition. Table 6.3 compiles the HB:HV content calculated from the production fluxes (experimental and predicted by segregated FBA) of each monomer for the five batch accumulation experiments (when more than 1 pulse was fed, the value presented corresponds to the average of all pulses of that batch). These differences in HB:HV content support the hypothesis suggest by Albuquerque et al. (2013) that the composition of the copolymer P(HB-co-HV) is not only associated with the VFA profile in fermented molasses composition but also with the populations present in the MMC.

Table 6.3: Experimental and segregated FBA predicted HB:HV content (C-mol HB:C-mol HV) of each population and the respective experimental fermented molasses feed composition.

Batch Experiment	Measured	Segregated FBA predicted			FM composition (C-mol basis)
		Azoarcus	*Thauera*	*Paracoccus*	
	HB:HV	HB:HV	HB:HV	HB:HV	Ac/Prop/But/Val/FA
FM I	**78:22**	**90:10**	**-**	**80:20**	**75/10/15/0/0**
FM II	**79:21** (7:7)	**82:18** (1:1)	**69:31** (1:1)	**92:8** (11:11)	**42/9/19/4/25** (2/3/2/0/0)
FM III	**67:33** (6:6)	**72:28** (6:6)	**61:39** (7:7)	**65:35** (9:9)	**50/23/9/0/18** (1/1/0/2/1)
FM IV	**77:23** (2:2)	**92:8** (9:9)	**82:18** (6:6)	**85:15** (1:1)	**41/6/30/4/19** (1/0/0/0/1)
FM V	**88:12** (3:3)	**84:16** (2:2)	**45:55** (6:6)	**87:13** (3:3)	**52/9/17/4/18** (3/1/1/1/4)

The values represent the average for all the pulses within a batch and between brackets is the respective standard deviation.

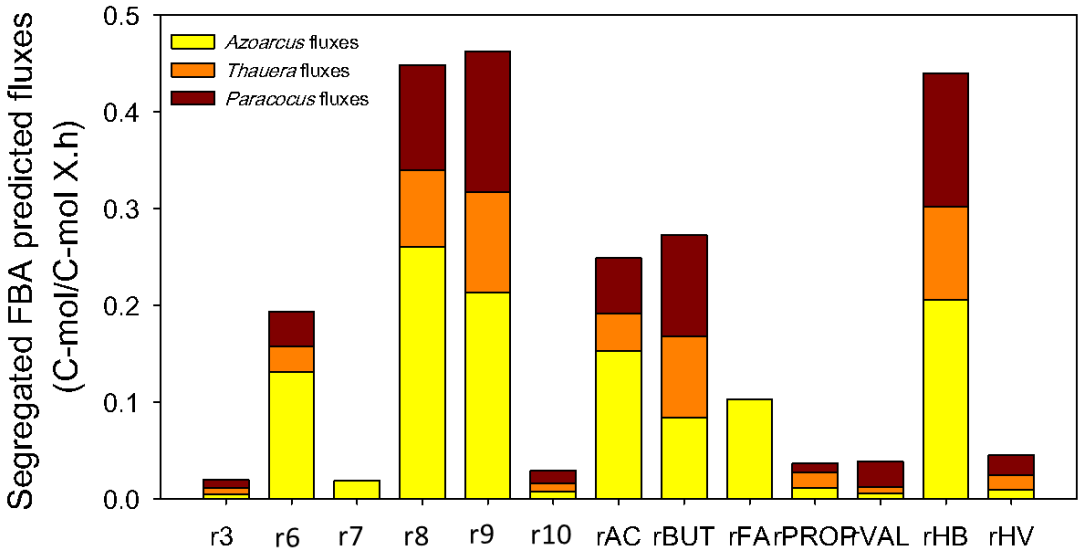


Figure 6.3: Metabolic flux distribution for each population calculated by segregated FBA. The results presented are for the 2nd pulse of FM IV batch experiment.

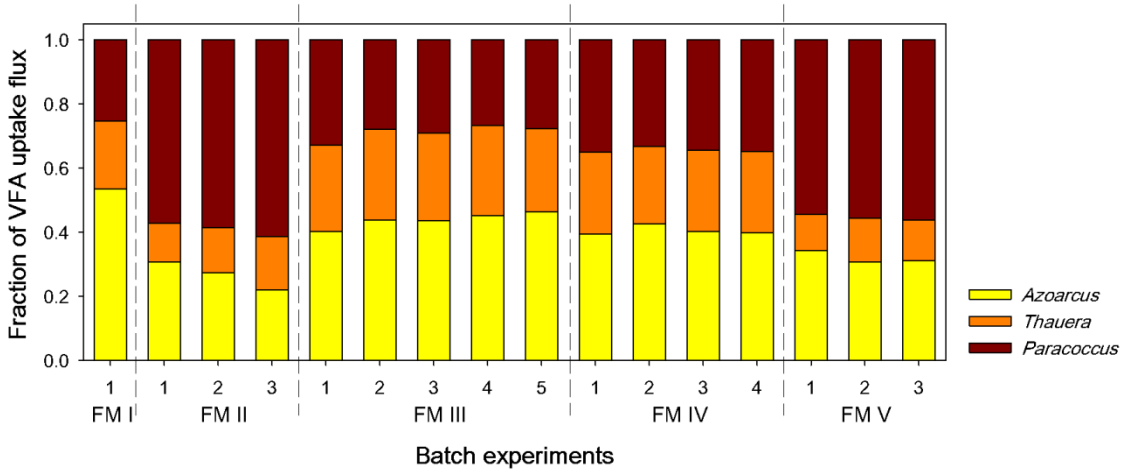


Figure 6.4: Distribution of overall VFA uptake flux for each population calculated by segregated FBA in all experiments.

6.4. Conclusions

In the present work, a segregated FBA method was developed and applied to a PHA-accumulating MMC. The main motivation was to develop a mathematical tool that enables to characterize the metabolic heterogeneity of an MMC composed by different populations. The identified metabolic objective was the minimization of the TCA activity for each of the populations present in the MMC. The rationale behind such metabolic objective is the feast and famine reactor operation mode to enrich the MMC in bacterial species that minimize respiration and therefore maximize PHA storage. Apart from the typical stoichiometric and reaction irreversibility constraints, which were the same for all the populations, FISH and MAR-FISH data derived constraints were included in the FBA, which ultimately confer the segregated character of the method. The results show that the segregated FBA could predict the average PHA storage flux and the respective HB:HV monomeric composition with high accuracy for all experiments performed and slightly better than the nonsegregated FBA. FBA predicted fluxes are highly concordant with MFA estimated fluxes, with the additional advantage of being able to differentiate the metabolisms of the different populations. This can be important to predict the performance of highly dynamic communities. From the differences in HB:HV content calculated for each population, it can be hypothesized that the average composition of the copolymer P(HB-co-HV) might be controlled not only by the VFA profile fed to the reactor but also by the population structure present in the MMC.

7 Dynamic metabolic modelling

In this chapter, we present a dynamic metabolic model that describes the uptake of complex mixtures of volatile fatty acids (VFA) and respective conversion into PHA by mixed microbial cultures (MMC). This model builds upon a previously published flux balance analysis model (Pardelha et al., 2012 - Chapter 5) that identified the minimization of TCA cycle activity as the key metabolic objective to predict PHA storage fluxes and respective composition. The model was calibrated either with experimental data of PHA production from fermented sugar cane molasses or from synthetic mixtures of VFA. All PHA production experiments were performed using a MMC selected with fermented sugar cane molasses under feast and famine regimen. The model was able to capture the process dynamics denoted by an excellent fit between experimental and computed time profiles of concentrations with the regression coefficients always above 0.92. The introduced VFA uptake regulatory factor reflects the decrease of acetyl-CoA and propionyl-CoA available to TCA cycle in conformity with the hypothesis that the minimization of TCA cycle is a key metabolic objective for MMC subjected to feast and famine regimen for the maximization of PHA production.

The contents of this chapter were adapted from the publication: Pardelha F, Albuquerque MGE, Reis MAM, Dias JML, Oliveira R. 2013. Dynamic metabolic modelling of volatile fatty acids conversion to polyhydroxyalkanoates by a mixed microbial culture. New Biotechnology. DOI:10.1016/j.nbt.2013.06.008 [1]

7.1. Introduction

Polyhydroxyalkanoates (PHA) are biopolymers that fulfil the requirements of a green bioplastic as opposed to conventional petroleum-based plastics. Their physico-chemical properties are similar to polypropylene and polystyrene with the advantages of being biodegradable, biocompatible and produced from renewable sources.

Nowadays, commercial PHAs are produced using pure or recombinant cultures and well-defined synthetic media with single substrates at high production costs. The use of waste materials as substrate is a strategy to decrease PHA production costs pursued by many researchers (Albuquerque et al., 2007 and reviewed by Serafim et al., 2008). Several authors investigated different reactor configurations and operating conditions (Albuquerque et al., 2010b; Johnson et al., 2010a, 2010b; Villano et al., 2010), while others focused on waste-based substrates and their influence in the process and/or in the PHA properties (Albuquerque et al., 2011; Bengtsson et al., 2008; Jiang et al., 2011b; Moita and Lemos, 2011). Other studies addressed the microbial characterization of the MMC (Albuquerque et al., 2013; Lemos et al., 2008; Pisco et al., 2009).

Mathematical modelling of PHA production by MMC has also received considerable attention (Dias et al., 2005, 2008; Jiang et al., 2011a; Johnson et al., 2009b). Over the last years the complexity of models has increased considerably. Beun and co-workers (2000a) developed the first consistent metabolic model, stemming from (Beun et al., 2000c; Beun et al., 2002), which was able to describe polyhydroxybutyrate (PHB) production from acetate. Other studies (Dias et al., 2005; Johnson et al., 2009b) proposed modifications of Beun models but always for the single substrate scenario.

The production of co-polymers of PHA, namely P(HB-co-HV), is of high interest because a more complex substrate could be used and its mechanical properties are better or similar than the homopolymer PHB (Albuquerque et al., 2011; Arcos-hernández et al., 2013). Dias et al. (2008) extended their previous single substrate (acetate) model for the case of two substrates (acetate + propionate) to describe the production of P(HB-co-HV) co-polymers. Later on, Jiang et al. (2011a) improved that model to describe cell growth and co-polymers production simultaneously.

Waste-based feedstocks normally have complex composition and comprise several substrates that can be metabolized by MMC to PHA. Currently, there is no dynamic metabolic model that describes multiple VFA conversion to PHA. Our previous model (Dias et al., 2008) cannot be directly extended for more than two VFA. With that model structure, more than 2 VFAs would

result in an undetermined system of material balances, which would require the modelling of additional kinetic laws for VFAs uptake. In a previous study, Pardelha et al. (2012 - Chapter 5) applied flux balance analysis (FBA) to describe the conversion of complex mixtures of volatile fatty acids (VFA) to PHA, namely fermented sugar cane molasses. FBA is a constraint-based modelling approach that can be applied to underdetermined metabolic networks based on the optimization of a predefined objective function under the constraints of reactions stoichiometry, reactions irreversibility and experimental flux data. On the basis of published results about PHA regulation (summarized in (Pardelha et al., 2012 - Chapter 5)), the objective function defined was the minimization of TCA cycle fluxes to optimize the carbon flux available for PHA production. This approach enabled to accurately predict PHA storage fluxes and also the respective HB:HV composition, from measurements of VFA uptake fluxes. This result corroborates the hypothesis that MMC selected under the feast and famine feeding regimen maximizes carbon usage for PHA storage.

FBA is a static modelling method that cannot be used to predict PHA production dynamics. The aim of the present study is to extend the previous FBA model to a dynamic formulation able to describe the uptake of complex mixtures of volatile fatty acids (VFA) and respective conversion to PHA along batch time. To accomplish this, the minimization of TCA cycle activity (Pardelha et al., 2012 - Chapter 5) was implemented through a regulation factor that controls the flux of carbon through the main metabolic pathways (Pardelha et al., 2012 - Chapter 5). The definition of this regulation factor was based on the cybernetic approach proposed by Ramakrishna et al. (1996) and Venkatesh et al. (1997) for multiple substrates uptake regulation.

7.2. Metabolic model

7.2.1. Metabolic network

The metabolic network used in this Chapter is described in Chapter 4.

7.2.2. Connectivity, thermodynamic and kinetic constraints

Similarly with previous published work (Pardelha et al., 2012 - Chapter 5), the metabolic network is defined by 10+k reactions (k stands for acetate, propionate, butyrate, valerate, even and odd unknown carbon sources (E_FA and O_FA)), 6 intracellular metabolites (acetyl-CoA, propionyl-CoA, acetyl-CoA*, propionyl-CoA*, $NADH_2$, ATP), 2+k input substrates (O_2, ammonia, all carbon sources) and 4 end-products (biomass, PHB, polyhydroxyvalerate (PHV) and CO_2) (see Table 3.3). The steady-state material balances for the 6 intracellular metabolites (represented by a

system of algebraic equations published in (Pardelha et al., 2012 - Chapter 5)) were considered as connectivity constraints.

All metabolic reactions but R_9 and R_{10} are irreversible. These two reactions comprise the β-oxidation pathway of longer chain VFA into acetyl-CoA and propionyl-CoA. In case of PHA precursors synthesis overflow, the excess is converted back to acetyl-CoA and propionyl-CoA via β-oxidation. Thus, $r_i \geq 0, \forall_{i \neq 9,10}$.

The system of equations resulting from material balances is undetermined (10 – 6 + k = 4 + k degrees of freedom) and thus additional kinetic constraints for VFA uptake, PHA monomers formation and maintenance were formulated to allow all network fluxes quantification:

The uptake of the i^{th} VFA (r_i) is limited by its concentration with $r_{i,max}$ the maximum uptake rate and K_i the half saturation constant.

$$r_i' = r_{i,\max} \cdot \frac{S_i}{S_i + K_i} \qquad \text{where} \quad i = \{E\_FA, O\_FA\} \tag{7.1}$$

The fluxes of acetyl-CoA* and propionyl-CoA* production (r_9 and r_{10}, respectively) are inhibited by the intracellular PHA content. Symbols $r_{9,max}$ and $r_{10,max}$ are the maximum specific rates for the synthesis of each precursor. $f_{PHA,max}$ and α are the maximum intracellular PHA fraction and the saturation order constant, respectively (see Beun et al., 2002 and Dias et al., 2005).

$$r_9' = r_{9,\max} \cdot \left(1 - \left(\frac{f_{PHA}}{f_{PHA,\max}}\right)^{\alpha}\right) \tag{7.2}$$

$$r_{10}' = r_{10,\max} \cdot \left(1 - \left(\frac{f_{PHA}}{f_{PHA,\max}}\right)^{\alpha}\right) \tag{7.3}$$

According to Ren et al. (2009), fatty acids oxidation into acetyl-CoA* and propionyl-CoA* is likely to be controlled by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios. PHA pathway is known to be a cell mechanism for channelling acetyl-CoA surplus and for regenerating NADH into NAD^+ (Senior and Dawes, 1973). Under PHA saturation, PHA content controls PHB synthase activity and this pathway slows down (Jung et al., 2000), causing an accumulation of these two metabolites. NADH accumulation also reduces TCA activity through the inhibition of citrate synthetase and isocitrate dehydrogenase activities according to Senior and Dawes (1971). The regulation mechanisms of these two process cause an accumulation of acetyl-CoA and NADH and consequently an increasing of [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios.

Despite the regulation mechanism for PHA production and degradation being relatively known, the inherent contribution of each ratio on substrates and products kinetics remains unclear. Therefore, to cope with the uncertainty about these mechanisms, a cybernetic variable, Φ, was defined following the modelling framework proposed by Ramakrishna et al. (1996) and Venkatesh et al. (1997) for microbial growth in a multiple substrate environment. When substrates are competing, multiple control parameters must be considered. In our case there is no experimental evidence of competition between different VFA. Thus a single control parameter was considered given by the ratio between the required substrate flux for cell maintenance plus actual biosynthetic capacity ($r_{S,req}$) and the total substrate uptake capacity $r_S = \sum_i r'_i$.

$$\Phi = \frac{r_{S,req}}{r_S} \tag{7.4}$$

$r_{S,req}$ is obtained by the analytical solution of the system of equations resulting from material balances (see Chapter 4).

Factor Φ can be viewed as a mathematical representation of the cell regulatory response to acetyl-CoA and/or propionyl-CoA overflow:

$$\begin{cases} r_i = r'_i \cdot \Phi, & i = \{E\_FA, O\_FA\} \;\; \Phi < 1 \\ r_i = r'_i \cdot \Phi^{-1}, & i = \{4,5,9,10\} \;\; \Phi \geq 1 \end{cases} \tag{7.5}$$

Whenever r_S is higher than $r_{S,req}$ (Φ < 1) the kinetic expressions of substrates are uniformly decreased to meet actual product synthesis capacity. Likewise, if r_S is lower than $r_{S,req}$ (Φ > 1) the product fluxes are uniformly decreased to meet the currently available resources.

Finally, the flux of maintenance on ATP (r_7) that represents the ATP consumed to sustain cellular function was fixed at the value proposed by Beun et al. (2002).

$$r_7 = r_{7,\max} = 0.02 \text{ mol-ATP.}\left(\text{C-mol.h}\right)^{-1} \tag{7.6}$$

Biomass growth kinetics was not considered in this work because biomass growth was negligible in the experiments performed. For more details about the kinetics of these reactions (r_4 and r_5) see Chapter 4 and Dias et al. (2008).

7.2.3. Numerical solution

For fermented molasses process there is 4 main substrates (acetate, propionate, butyrate and valerate) and other unknown substrates combined in fatty-acids (FA). Therefore, the metabolic model has 16 metabolic fluxes (unknown variables), 6 connectivity equality constraints (material balances) and 10 equality kinetic constraints (Eqs. 7.1 – 7.6) (note that for specific case of fermented molasses the indexed Eq. 7.1 actually represents 6 equations – acetate, propionate, butyrate, valerate and odd and even fatty-acids). Thus the full set of constraints forms a determined and redundant system of algebraic equations with 1 degree of freedom. The 7 unknown fluxes were calculated by standard methods of metabolic flux analysis (see Pardelha et al., 2012 - Chapter 5 - and Stephanopoulos et al., 1998).

The consistency index (h), a statistical Chi-squared (χ^2) test, was used to check the consistency between assumed metabolic constraints (material balances and Eqs. 7.1 – 7.6) and measured flux vectors, under given flux error scenarios. More specifically, metabolic flux distribution estimations are said to be consistent when the weighted sum of flux errors (h) is below a given threshold χ^2 value (Wang and Stephanopoulos, 1983). In our case the threshold Chi-squared value is $\chi^2 = 3.84$ for a 95% confidence level and 1 degree of freedom. For more details about the consistency index method see Stephanopoulos et al. (1998).

7.2.4. Kinetic parameter estimation

Model parameters were estimated by non-linear weighted least-squares using the Levenberg-Marquardt algorithm and the confidence bounds were obtained by the estimate of standard deviations for a level of confidence of 95%. Detailed about this method are given elsewhere (Dias et al., 2005 and 2008).

7.3. Experimental data

The description of all material and methods and experimental data relevant for this work are presented on chapter 3 of this Ph.D. thesis. The batch experiments used in the present chapter were FMII, FM III and FMIV for fermented molasses and SFI and SFII for synthetic feed.

7.4. Results and discussion

7.4.1. Kinetic parameters estimation

MMC integrate a variety of microbial species, which compete for the carbon sources present in the feedstock. Thus, the observed metabolic activity is an 'average' of the metabolic activity of all the organisms present in the culture. In particular, the observed variability in PHA accumulation kinetics is largely determined by population dynamics induced by the feast and famine culture enrichment strategy. As such, local model parameters were estimated for each batch experiment individually because there is a considerable time lag between them with likely divergent MMC composition (Tables 3.2 and 7.1). The estimated parameter values and respective 95 % confidence intervals are presented in Table 7.1 for both groups of experiments using synthetic VFA mixtures or fermented molasses. Note the narrow confidence intervals denoting the high sensitivity of residuals to parameters and the high statistical confidence of the estimated parameter values. A few less sensitive parameters, namely K_S, K_N and α were preset at values estimated in previous works (Beun et al., 2002; Gujer et al., 1999).

Table 7.1: Parameter estimation results of fermented molasses enriched culture for the different feeding conditions. CI is the confidence interval and was obtained by error propagation of the metabolic model parameters.

Batch	FM II		FM III		FM IV		SF I		SF II	
Parameters	Value	CI	Value	CI	Value	CI	Value	CI	Value	CI
$r_{Ac,max}$	0.087	0.008	0.31	0.02	0.142	0.009	0.17	0.01	0.18	0.01
$r_{Prop,max}$	0.08	0.02	0.27	0.04	0.037	0.004	0.07	0.01	0.075	0.009
$r_{But,max}$	0.18	0.04	0.17	0.04	0.24	0.03	0.15	0.03	0.25	0.05
$r_{Val,max}$	0.037	0.009	0.01*	-	0.035	0.004	0.036	0.007	0.08	0.02
$r_{E\_FA,max}$	0.035	0.003	0.109	0.009	0.078	0.005	-	-	-	-
$r_{O\_FA,max}$	-	-	-	-	-	-	-	-	-	-
$r_{9,max}$	0.25	0.02	0.37	0.04	0.32	0.03	0.26	0.02	0.21	0.01
$r_{10,max}$	0.028	0.003	0.08	0.01	0.051	0.005	0.053	0.003	0.043	0.003
$f_{PHA,max}$	1.27	0.07	3.0*	-	2.2	0.2	2.3	0.1	3	1
δ	0.76	0.08	0.63	0.04	0.9	0.1	1.7	0.3	0.87	0.07
Fixed parameters: $r_{7,max}$ = 0.02 mol-ATP/(C-mol X.h)[1]; α = 3.84 [2]; K_s = 0.0625 mmol/L [2]; K_n = 0.56 N-mmol/L [2]; n = 2										

[1] Beun et al. (2002); [2] Dias et al. (2005, 2008)

7.4.2. Multiple VFA uptake kinetics

Experimental data confirm that the cells take up the 4 VFA simultaneously without any apparent competition effect between them (Figure 7.1). The data show, for instance, that when one VFA depletes the cells respond by increasing the uptake rate of the other VFA (results not shown). Also downstream product saturation or rate limitation shows a uniform down regulation effect in VFA uptake kinetics. Our hypothesis of VFA uptake regulation by acyl-CoA/CoA and NADH/NAD ratio seems to be consistent with these observations. Ren et al. (2009) suggested that the balance between PHA synthesis and fatty acid oxidation is controlled mainly by these ratios in cells. One of the key features of the model is the description of the down-regulation of VFA uptake and of PHA production based on the regulation factors Φ' and Φ''. Figures 7.2 j-l and 7.3 g-h show the regulation factor for substrate uptake ($\Phi' = \Phi$) and products synthesis ($\Phi'' = \frac{1}{\Phi}$) for complex fermented molasses and for synthetic VFA mixtures respectively.

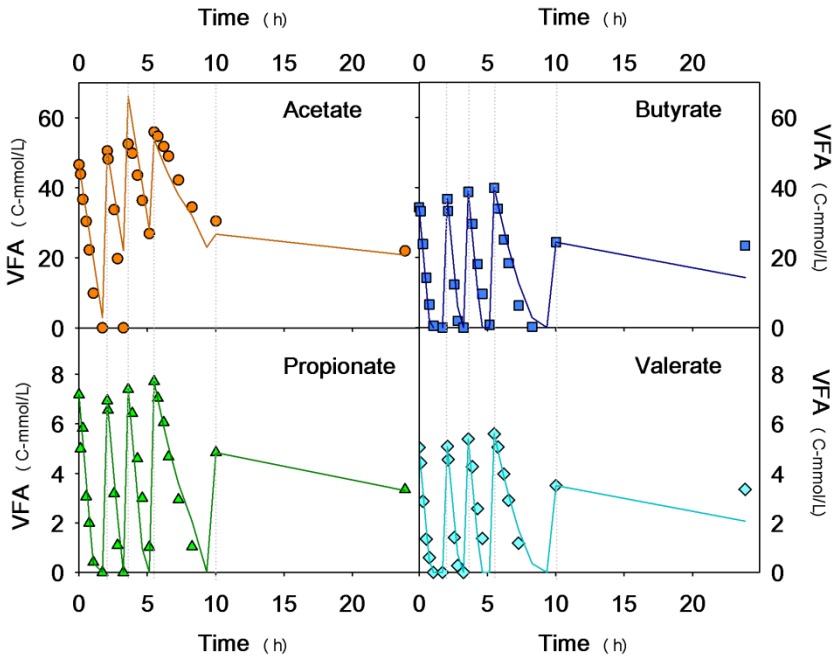


Figure 7.1: Modelling results of 4 VFA for experiment FM IV using fermented sugar cane molasses as carbon source. Symbols represent measured data and full lines represent the model predictions. Vertical dotted lines represent a new pulse fed.

As example, in experiment FMII (Figure 7.2 j) in the end of pulses one and two, limiting VFA concentrations are observed, which results in lower VFA uptake rates and lower PHA production rates (Φ'=1 and Φ''<1). Conversely, VFA uptake rates decrease under PHA saturation in the

end of pulse three (Φ'< 1 and Φ''=1). In both previous cases, the modelling error is slightly higher than when Φ'= Φ''=1 but the model accuracy remains acceptable. In general, it could be stated that regulation factor shows three different behaviours: I) under large VFA excess, Φ' decreases to values lower than 1 (ranging between 0.6-0.99) thereby constraining VFA uptake to the maximum acetyl-CoA and propionyl-CoA turnover capacity; II) under VFA limitation, acetyl-CoA and propionyl-CoA does not interfere with VFA uptake kinetics (Φ'= 1) but limit products formation to the actual acetyl-CoA and propionyl-CoA turnover (Φ''< 1) and III) under PHA saturation (last pulse of batch tests FM II, FM IV and SF I) fatty acids are severely down-regulated (Φ'< 0.6) by acyl-CoA/CoA and NADH/NAD ratio.

The described behaviour of the regulation factor reflects in the decrease of acetyl-CoA and propionyl-CoA available to TCA cycle. So, the results of our study are then coherent with the minimization of TCA cycle used as objective function on FBA based model (Pardelha et al., 2012 - Chapter 5) and reinforce the hypothesis how the metabolism of MMC subjected to feast and famine regimen is optimize to maximize PHA production.

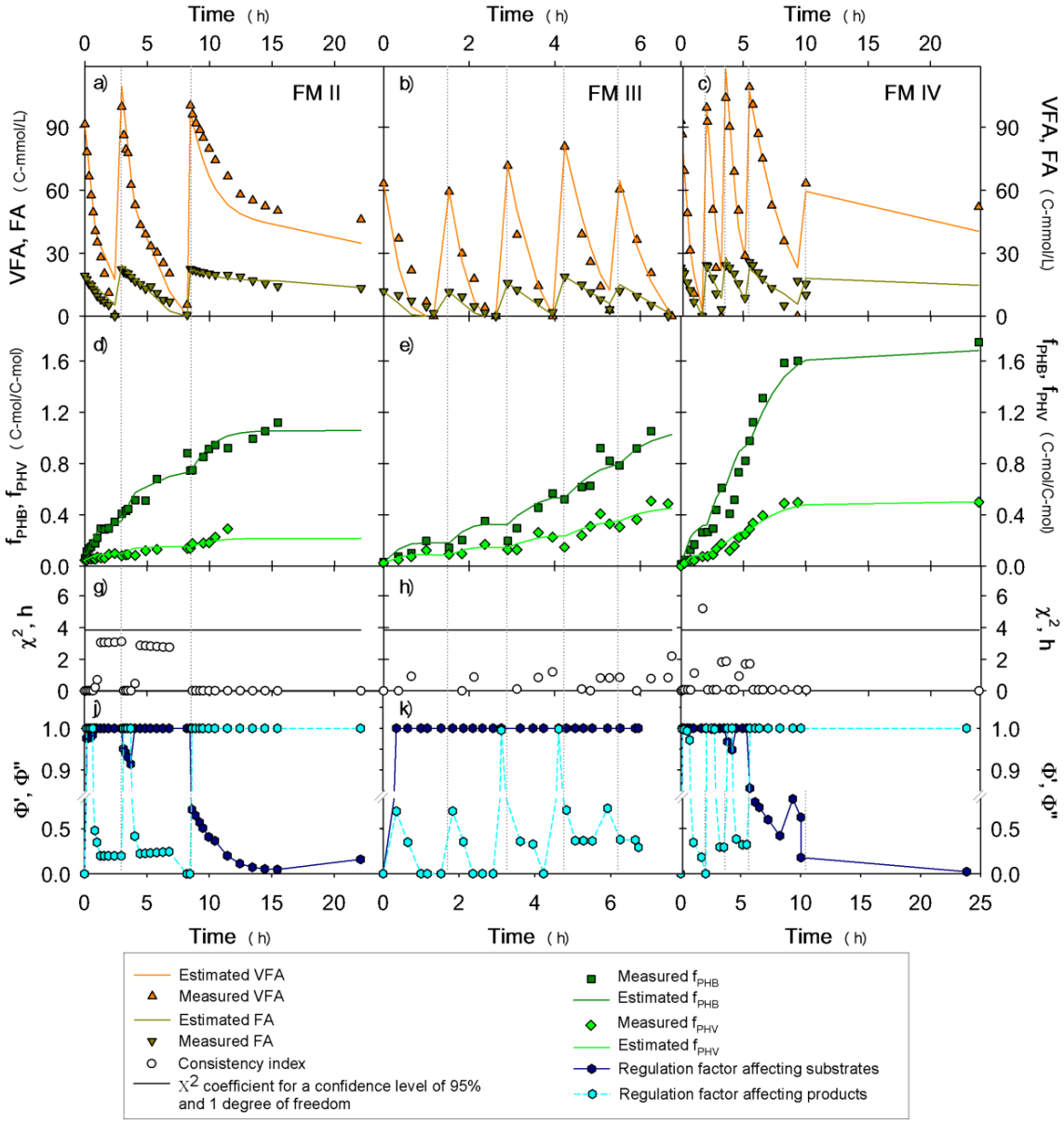


Figure 7.2: Modelling results for experiments FM II (a, d and g), FM III (b, e and h) and FM IV (c, f and i) using fermented sugar molasses as carbon source. Symbols in Figure 3 a-f represent measured data and full lines represent the model predictions. Symbols in Figure 3 g-l represent the consistency index (h) over time and the dotted lines represent the χ^2 433 coefficient for a confidence level of 95% and 1degree of freedom assuming an error in defined fluxes of 6%, 3% and 3% in experiments FM II, FM III and FM IV, respectively. Symbols in Figure 3 j-l represent regulation factor affecting substrates uptake and product synthesis. Vertical dotted lines represent a new pulse fed.

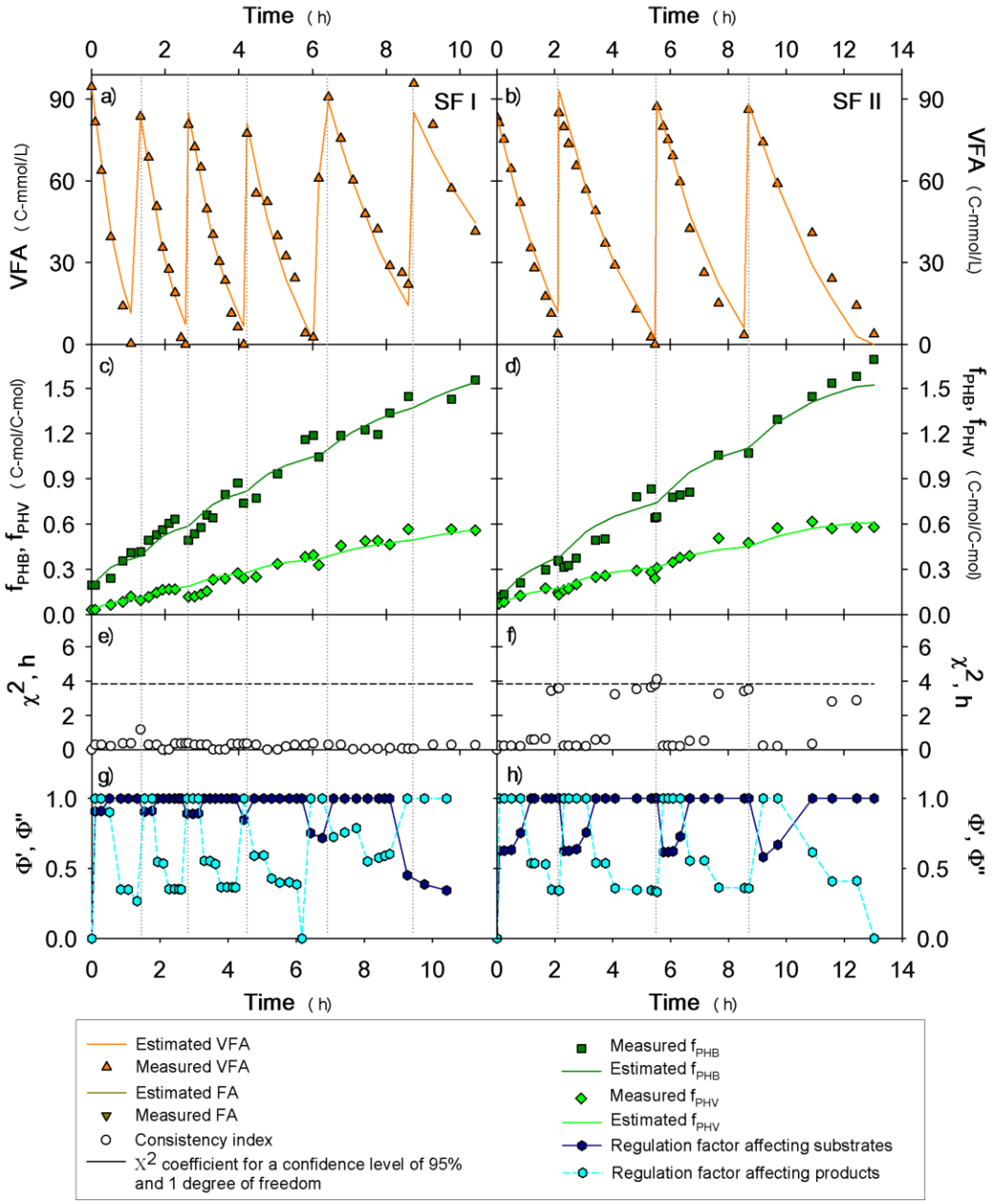


Figure 7.3: Modelling results for experiments SF I (a, c and e) and SF II (b, d and f) using synthetic feed as carbon source. Symbols in Figure 4 a-d represent measured data and full lines represent the model predictions. Symbols in Figure 4 e-f represent the consistency index (**h**) over time and the dotted lines represent the χ^2 coefficient for a confidence level of 95% and 1 degree of freedom assuming an error in defined fluxes of 2% in both experiments (SF I and SF II). Symbols in Figure 4 g-h represent regulation factor affecting substrates uptake and product synthesis. Vertical dotted lines represent a new pulse fed.

7.4.3. Dynamic simulation of batch experiments

All batch experiments were done following a pulse wise feeding strategy (see experimental data section and Chapter 3). This strategy prevents substrate limiting concentrations (Albuquerque et al., 2007) at the cost of adding a more dynamic concentrations time profile to the batch experiments. Nevertheless, the metabolic model developed in the present work was able to describe accurately these dynamics either for batch experiments using fermented sugar cane molasses and synthetic mixture of VFA.

The regression coefficients, R^2, were always above 0.92 showing that the model was able to capture the average process behaviour with very low bias thus providing an excellent fit to the data. Only the regression coefficients for FA are less satisfactory but even so above 0.77. This may be explained by the uncertainty around the FA metabolism and FA empirical formula (FA is not necessarily a carboxylic acid as assumed in the metabolic model). Figures 7.2 and 7.3 also show the comparison between modelled and measured state variables, namely VFA, FA, f_{PHB} and f_{PHV} for fermented molasses and synthetic VFA mixtures, respectively.

To further strengthen the statistical confidence of the metabolic model, the consistency index (h) was calculated for each batch experiment assuming arbitrarily low error scenarios in estimated fluxes. In the synthetic VFA mixture experiments, consistency is demonstrated for relative errors in estimated fluxes as low as 2% (see Figure 7.3 e,f) while in the complex fermented molasses consistency is verified for relative flux errors as low as 3%, except in case of FM II that was 6% (see Figure 7.2 g-i).

7.5. Conclusions

A MMC dynamic metabolic model capable to describe PHA production from mixtures of VFA has been developed in this study. The model was calibrated with experimental data of fermented sugar cane molasses and a synthetic mixture of VFA. The data showed that VFA uptake kinetics are essentially uncoupled. The model uses a cybernetic variable that regulates uniformly VFA uptake such as to adequate resources to metabolic needs. In general, good agreement between experimental data and model was obtained. Moreover, the $\chi 2$ consistency analysis showed that the assumed biological constraints are consistent with measured metabolic fluxes.

The results achieved also reinforce that the minimization of TCA cycle is how MMC subjected to feast and famine regimen optimizes their metabolism to maximize PHA production as suggested in (Pardelha et al., 2012 - Chapter 5).

8 *Conclusions and future work*

Polyhydroxyalkanoates (PHA) production by mixed microbial cultures (MMC) using fermented feedstocks is a process with a high degree of complexity mainly caused by the population heterogeneity and by complexity of these substrates. Several researchers studied these systems but most of works were focused on the optimization of operational conditions to achieve high PHA productivities and content. Computational system biology tools were developed and applied in this thesis to study the impact of population dynamics and the use of complex mixtures of substrates on microbial communities metabolism and consequently on process performance.

Constraint-based modelling was adopted in this work to prevent the use of frequently unknown kinetics for intracellular metabolites. Therefore, the first step was to define a metabolic network able to describe PHA production by MMC using complex mixtures of volatile fatty acids (VFA), which are the main compounds of fermented sugar cane molasses. While the previous published networks were only applied to mixtures of acetate and propionate, the current model copes with the presence of any arbitrary mixtures of VFA. Two generic reactions were added to describe the uptake of VFA with even and odd number of carbons. The number of parameters required to calculate cell growth and PHA production theoretical yields and maintenance increased but their expressions were successfully defined. This metabolic network was used as the starting point to develop computational tools over this Ph.D. thesis.

Although the metabolic network include both cell growth and PHA production processes, all the studies were only performed for the PHA production phase. However, the entire process was accounted on data interpretation and modelling results. The details of the entire process were described on Chapter 3 in order to support the interpretation of modelling results over the thesis. Summarizing, the process comprises the following stages: sugar cane molasses are converted into VFA by acidogenic fermentation (stage 1) and then the fermented sugar cane molasses are used as substrate in the phases for selection of PHA producing organisms (stage 2), performed under the feast and famine regimen, and PHA production (stage 3). For PHA production, the enriched MMC (collected at the end of the famine phase) is used as inoculum in a batch reactor to study the PHA storage performance by feeding consecutive pulses of either fermented sugar cane molasses or synthetic VFA mixtures. The measured VFA uptake fluxes were calculated for each pulse and then used as inputs to the metabolic models.

The Metabolic Flux Distribution (MFD) for several PHA production batch experiments was calculated using Flux Balance Analysis (FBA) and the metabolic network. The goal was to better understand the effect of using complex mixtures of VFA on PHA storage. PHA storage fluxes and HB:HV composition were successfully predicted from measured VFA uptake fluxes by

applying the minimization of TCA cycle fluxes as objective function. This result supports the hypothesis that the feast and famine feeding regimen promotes a more efficient utilization of carbon for PHA storage instead of respiration. It was also concluded that PHA production fluxes are higher when the same substrate is used in both selection and PHA production stages. Higher intracellular level of $NADH_2$ also increases PHA fluxes as consequence of its down-regulation effect on TCA cycle fluxes.

Population dynamics has also a critical role on the efficiency of PHA storage by MMC. The segregated FBA method was developed aiming at the characterization of the metabolic heterogeneity of MMC composed by different populations. FISH and MAR-FISH-derived data were added as constraints along with the stoichiometric and reaction irreversibility constraints, to confer the segregated character to the model. The application of the segregated FBA improved the prediction of the average PHA storage flux and HB:HV monomeric composition in comparison to the previous FBA. The fluxes predicted by this model were also highly concordant with MFA estimated fluxes for the different populations. It was hypothesized in this study that the VFA composition and the population distribution of MMC might control the average composition of the copolymer P(HB-co-HV).

The previous metabolic models were applied individually to each pulse of batch experiments. However, it was also important to assess the cumulative effect of each additional pulse on the metabolic state of MMC. Therefore, a dynamic metabolic model capable to describe PHA production by MMC from mixtures of VFA was developed. This model included a regulatory factor for VFA uptake and PHA production in order to adequate resources for the metabolic requirements. The main conclusions of this study were that VFA uptake kinetics are essentially uncoupled and that TCA cycle fluxes were minimized over the enrichment process under feast and famine regimen in order to maximize PHA production. The latter results support the conclusions obtained in previous Chapters.

Computational systems biology tools developed over this thesis can be a starting point for future works, namely to study the effect of operational conditions on cell growth. Currently, this is the major bottleneck that hinders the increasing of PHA productivity in MMC. In this case, the number of unknown fluxes will be higher and the inclusion of additional measured fluxes, such as oxygen consumption and CO_2 production, will be mandatory for the complete identification of the metabolic flux distribution. A metabolic model able to describe the simultaneous production of biomass and PHA will be also helpful to better understand, at the metabolic point of view, the transition between cell growth and PHA production. This study will enable to optimize the selection stage aiming at achieving a culture with both high cell growth and high PHA

productivity. Despite the intensive research activity in this area, the optimal operational conditions that leads to an enriched culture with such characteristics was not reported so far. Certainty, these findings will be a landmark on PHA production by MMC.

The identification of the $NADH_2$ and ATP dynamics at different enrichment conditions can highlight additional strategies for process optimization. In the present, it was verified that $NADH_2$ has a major role on PHA storage. The present metabolic network can be applied to estimate ATP and $NADH_2$ pools from experimental data obtained by different research groups in order to understand how the operational conditions affect these pools and consequently process performance. Furthermore, the ATP and $NADH_2$ pools can be also correlated with several process parameters (cell growth and PHA production yields, maintenance coefficients, PHA storage capacity or PHA productivity) using chemometrics techniques such as Principal Component Analysis (PCA) or Partial Least Squares (PLS). Preliminary studies performed confirmed the high impact of the pools of these metabolites on process performance, namely on growth rate and PHA productivity.

The developed models can be further extended into a metabolic model that accounts with population dynamics. This segregated and dynamical model will enable to elucidate the potential interactions between the populations present in the MMC. The resolution of the model can be further improved by using as constraints data from “omics” techniques able to identify key quorum sensing molecules responsible for the “communication” between populations.

Finally, metabolic models can be also applied on process optimization, control and monitoring studies by using easily accessible measurements such as pH, dissolved oxygen, dissolved carbon dioxide, on-line respirometry and on-line titrimetric analysis.

As summary, this Ph.D. thesis provided an important contribution to understand PHA production by MMC using real feedstock and contributed with some clues for subsequent process optimization. More specifically, the potential of using computational systems biology tools to support the interpretation of the experimental data and to unravel possible metabolic bottlenecks was undoubtedly demonstrated in this study.

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